INHIBITION OF SPERMATOGENESIS IN RABBITS WITH TESTOSTERONE FILLED POLYDIMETHYLSILOXANE IMPLANTS

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TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER I

INTRODUCTION

The "Human World Population", that is, the number of hands available for work and the number of mouths to feed is the basic determinant of economic activity. However, world prosperity depends not only on the number of people alive at any given time but also on how efficiently they work and how much income they have to spend. It is people who produce and consume, build and destroy, procreate and die. Moreover, it is the wisdom and efficiency with which they carry out these activities that keeps the world's economic machinery running and thus maintains high standards of living in sophisticated societies.

During most of man's existence on earth, high fertility was necessary if the race was to survive. Throughout the world prior to 1600 AD, population never grew faster than 0.3 percent per year except where virgin land was being settled. The growth rate did not exceed 0.5 percent a year until after 1800 AD.

Prior to World War II, life expectancy for people living in the underdeveloped countries was low and parents who wanted to be assured of having children to care for them in their old age attempted to raise large families.

Following World War II, technological developments of Europe and North America were introduced on a large scale into the world's more backward regions. Techniques for improving public health were widely

adopted. Life expectancies in some areas of the underdeveloped world have increased to a level only moderately below that of the United States thus high death rates no longer cancel out high birth rates.

The dimensions of the population problem today are of great magnitude. Half of the world's present population has been born since the end of World War II. The present population growth rate is almost 2 percent per year, adding approximately 70 million people each year. This means adding another New York City to the world's population every 6 weeks.

If the population growth is allowed to continue unabated, the prime concern will be enough food to sustain human life. Considering the current level of agricultural technicology food supplies may be maintained to support the world population until the year 2025 A.D. Beyond this date the outlook is bleak.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Overpopulation is the greatest problem facing mankind today. Population growth in the United States emerged as a prominent concern with urban and environmental deteriorations in the United States and with increased world population pressures.

Systemic methods of contraception have been developed for the female. However, very little effort has been expended in the development of systemic contraceptive measures in the male.

Spermatogenesis is a lengthy process of cell division and maturation resulting in the formation of spermatozoa. This process is interrupted by exposure to heat, irradiation, cadmium, immunologic factors, certain chemical agents and some gonadal hormones. However, most of these factors cannot be used as effective contraceptive measures because of irreversible changes in the germinal epithelium or because of deleterious side effects. The simplest method of inhibiting spermatogenesis with the minimum of deleterious side effects might involve intereference with pituitary regulation of testosterone secretion by the interstitial cells of Leydig. However, questions such as: what hormonal factors regulate the production of spermatozoa by the germinal epithelium and what factors are known to inhibit spermatogenesis remain to be answered.

The Regulation of Spermatogenesis

The seminiferous tubules of the testis are lined with an epithelium from which spermatozoa are proliferated. Spermatogonia lying next to the basement membrane, undergo a series of mitotic divisions leading to the formation of primary spermatocytes. These undergo the first meiotic division and give rise to cells called secondary spermatocytes. The latter begin the second meiotic division almost immediately and produce smaller haploid cells which are called spermatids. The process of transformation of spermatogonia to spermatids, ready to be released into the lumen of the seminiferous tubule, is called spermatogenesis.

The germinal epithelium carries out three principal processes: 1) increase in number of cells by mitosis, 2) reduction in the number of chromosomes by meiosis, and 3) the production of testicular spermatozoa from spermatids by spermiogenesis.

Although it is known that complete spermatogenesis requires specific hormones, the endocrine regulation of the germinal epithelium is complex and general aggreement has not been reached regarding the nature of the hormones required (Hall, 1970).

Influence of Hypophysectomy on Spermatogenesis

Spermatogenesis fails in the hypophysectomized animal or man (Greep and Fevold, 1937) (Lipsett et al., 1956). A number of investigators (Smith, 1927) (Greep and Fevold, 1937) (Woods and Simpson, 1961) (Lostroh, 1963) realized that pituitary ablation caused gonadal atrophy. However, difficulty was encountered in attempts to separate the effect of damage inflicted by the ablation procedure to the

pituitary, hypothalamus, and adjacent brain tissue. Smith's (1927, 1930) classical experiments demonstrated atrophy of the testes and cessation of spermatogenesis in male rats following hypophysectomy. Moreover he showed that the pronounced atrophy of the testes, and accessory sex organs was restored to near control levels by daily administration of anterior pituitary extract.

Extensive investigations (Greep et al., 1936, 1937) in succeeding years confirmed Smith's observations, and led to the hypothesis that pituitary gonadotrophic factors may play specific roles in maintaining testis function. This hypothesis was widely accepted and led to the concept follicle stimulating hormone (FSH) controlled the spermatogenic process and leutinizing hormone (LH) controlled testosterone production by Leydig cells.

Greep's formulation of the above concept resulted from using crude preparations of FSH and LH and thus is suspect.

Woods and Simpson (1961) and Lostroh (1963) have questioned the exact role of FSH and LH in regulating testis function. Administration of anterior pituitary extracts to hypophysectomized rats will restore and maintain spermatogenesis, (Smith, 1936) (Woods and Simpson, 1961). Similarly, ICSH, (Simpson et al., 1944) or androgens, (Nelson and Gallagher, 1936) have been shown to maintain spermatogenesis in hypophysectomized animals. The concept that FSH maintains germinal epithelium and ICSH stimulates the Leydig cells has been challenged by Lostroh et al., (1963). Lostroh's group reported repair of the germinal epithelium in hypophysectomized rats given ovine FSH plus one milligram or less of ICSH per day, but concomittant treatment with antiserum to ICSH prevented spermatid formation. The authors concluded that FSH may play

a role in the formation of spermatogonia and primary spermatocytes, but suggest that the formation of spermatids requires both hormones.

Steinberger and Duckett, (1965) injected male rats with estradiol benzoate or testosterone propionate daily from birth. The weight of testes from animals receiving estrogen for periods up to two months was suppressed. No increase above that found in normal thirty day old, prepuberal animals was found. The first wave of spermatogenesis commenced at the expected time, but proceeded only to the stage of pachytene primary spermatocytes. In no case was completion of the meiotic division observed.

Weight of testes from testosterone treated animals was suppressed, but to considerably less extent than that of estrogen treated animals. Spermatogenesis in the testosterone treated animals commenced at the expected time and proceeded normally up to primary spermatocytes, but spermatid appearance was significantly delayed and complete maturation did not occur, even in animals three months of age. This work suggests spermatogenesis can commence in rats receiving doses of estrogen or testosterone which inhibit pituitary gonadotrophins (Ludwig, 1950), but will not proceed to mature spermatozoa. Confusing results reported on restoration of spermatogenesis in hypophysectomized animals by testosterone may be contributed to, by: 1) incomplete hypophysectomy, allowing a low output of pituitary gonadotrophins in some animals. This constituted up to 25 percent of the experimental animals in one study reported by Lostroh (1963); 2) time elapsing from hypophysectomy and initiation of replacement therapy; and 3) age of the animals used in the experiments.

Although the exact roles played by FSH and LH in mammalian

spermatogenesis are confused, there is little doubt that these gonadotrophins are necessary for complete spermatogenesis to occur. It is suspected that prolactin and growth hormone may play important roles in spermatogenesis. Growth hormone administered simultaneously with ICSH enhanced androgen secretion in hypophysectomized rats as evidenced by increased testicular and prostate gland weights in rats (Woods and Simpson, 1961) (Lostroh et al., 1958). Growth hormone administered simultaneously with FSH and testosterone increased the testicular weight 57 percent over that obtained with FSH and testosterone (Lostroh, 1969). Hypophysectomized rats receiving ICSH, growth hormone, and prolactin showed larger increases in testis weight than animals receiving ICSH and growth hormone. However, ICSH plus prolactin treatment did not increase testicular weight as much as ICSH plus growth hormone. These latter results suggest that growth hormone permits prolactin to exert an action on the testis. Thus it appears that several trophic hormones from the pituitary are involved in the regulation of spermatogenesis.

Influence of Testosterone Upon Spermatogenesis

The male sex hormone, testosterone, is synthesized and secreted by the intersititial cells of Leydig which are interspersed among the seminiferous tubules. Christensen and Mason (1965) have shown that most testosterone produced within a testis is from the interstitial tissue, with only a small amount being produced by the seminiferous tubules.

The effect of testosterone on spermatogenesis has been elucidated by experiments testing: 1) the effect of testosterone in hypophysectomized animals, 2) the effect of testosterone in animals with impaired release of pituitary gonadotrophins and 3) the effect of testosterone on the hypothalamus.

Effect of testosterone in Hypophysectomized Animals. The ability of testosterone to maintain or initiate and maintain spermatogenesis in hypophysectomized animals has been studied by many investigators. Hamilton and Leonard (1938) reported that daily injectins of 500 µg of testosterone propionate (TP) initiated immediately after hypophysectomy resulted in scrotal testes that were ninety-four percent heavier than those in untreated hypophysectomized control rats. Histological examination of control testes revealed that there were few or no spermatozoa although spermatids were present in the seminiferous tubules. The seminiferous tubules in the hypophysectomized rats receiving TP treatment daily for six weeks following surgery were well developed, but spermatids tended to clump and mitoses were fewer in number. When the initiation of TP treatment was delayed nine to fifty-one days after hypophysectomy, well developed tubules were never observed.

Woods and Simpson (1961) reported no maintenance of spermatogenesis in male rats hypophysectomized at forty days of age and treated with 10 µg testosterone propionate (TP) daily for fifteen days. However, in the same animal model, 50 µg TP daily for fifteen days produced spermatids in forty percent of the rats tested.

Woods and Simpson (1961) also reported on the ability of testosterone to repair the spermatogenic process in rats hypophysectomized at forty days of age with initiation of TP treatment delayed fourteen days past hypophysectomy. Three levels of the hormonal steroid (10, 50, and 250 µg per day) were given to three groups of rats for twenty-eight days. Only the animals receiving the 250 µg per day dosage of TP produced any spermatids. Moreover, the 250 µg TP daily returned testicular weights to only twenty-two percent of normal unoperated rats the same

age, while increasing the weights of the ventral prostate and seminal vesicles 260 percent and 665 percent above normal, respectively. These results suggest that the level of testosterone needed for maintenance of the germinal epithelium is much higher than the level needed for maintenance of accessory sex organs.

Ludwig (1950) reported the results of experiments testing the effect of testosterone on the seminiferous epithelium of rats hypophysectomized at thirty days of age. One testis was removed at the time of hypophysectomy and the other removed following thirty days of TP administration (1.0 mg/day). Histological examination of testes removed at the time of hypophysectomy (thirty days of age) revealed early spermatids predominating in the tubules. Seminiferous tubules of hypophysectomized controls at sixty days of age showed a marked decrease in diameter and degeneration of the germinal epithelium to spermatogonia and spermatocytes. Fifty percent of the animals receiving 1.0 mg TP daily, post-hypophysectomy, to sixty days of age maintained seminiferous tubule size and some tubules contained spermatids and spermatozoa.

Boccabella (1963) reported reinitiation and restoration of spermatogenesis with TP after a long-term posthypophysectomy period. Treatment of hypophysectomized male rats with 3 mg per day of TP initiated 65 days after surgery and contineud for 90 to 110 days, restored spermatogenesis in approximately 33 percent of the animals tested.

The studies reviewed above utilized hypophysectomized animals. The replacement therapy was initiated immediately or delayed allowing the germinal epithelium to degenerate. There are obvious problems with the hypophysectomized animal model including: 1) incomplete removal of

all gonadotrophin producing tissue (Lostroh, 1963); 2) unfavorable internal environment resulting from the removal of the trophic hormones regulating the functions of other endocrine glands, and 3) the loss of sensitivity of the testis to exogenously administered hormones, depending upon the time of initiation of treatment following hypophysectomy. In light of the above reservations, other experimental approaches have been used to determine the effect of testosterone upon spermatogenesis.

Effect of Testosterone in Animals in Which the Secretion of Pituitary Gonadotrophins Has Been Inhibited. Steinberger and Duckett (1965) investigated hormonal requirements for the initiation of spermatogenesis in newborn rats and studied the factors required for its completion. They demonstrated that daily administration of estradiol benzoate from birth, in doses that suppress pituitary gonadotrophins to undetectable levels, does not prevent initiation of the spermatogenic process. The first wave of spermatogenesis proceeds to the stage of late pachytene spermatocytes. Continued estradiol treatment results in the failure of subsequent waves of spermatocytes to form. Steinberger and Duckett (1967) reported results of the administration of testosterone to 15 day old rats receiving estradiol daily from birth. Testosterone injections were started (2 mg per day) at 15 days of age thus the animals received both testosterone and estradiol from 15 to 65 days of age. Although the mean testicular weight at 60 days of age was far below that of normal 60 day old animals the mean testicular weight was 580 percent greater than that of rats receiving no testosterone therapy. Spermatogenesis proceeded to midpoint in most seminiferous tubules and the prostate and seminal vesicles were stimulated.

Karla and Prasad (1967) reported that daily administration of

clomiphene (1- p (beta-diethylaminaethoxy-phenyl - 1, 2-diphenyl-2-chloroethylene) to immature rats beginning on day 22, caused an arrest of spermatogenesis at the primary spermatocyte stage. Two hundred micrograms of testosterone propionate administered daily from day thirty, to clomiphene-fed rats, advanced spermatogenesis from the primary spermatocyte stage, present at initiation of TP treatment, to the spermatid stage (step 1-7). These data were interpreted to mean that TP exerts a stimulatory action on the early phase of spermatogenesis. Administration of 0.5 to 1.0 mg per day of TP to clomiphene fed rats resulted in the occurrence of mature spermatozoa in the seminiferous tubules suggesting that high doses of testosterone propionate facilitates the advance of spermatogenesis from the primary spermatocyte stage to mature spermatozoa in the absence of gonadotrophic hormones.

Effect of Testosterone on the Hypothalamus. Davidson (1967), reviewed hypothalamic control of testosterone secretion and spermatogenesis in testis. He concluded the integrity of the hypothalamic median eminence is essential for maintenance of spermatogenesis by pituitary hormones. This region appears to contain the receptors sensitive to changes in circulating testosterone levels, since implantation of crystalline testosterone into this region, but not in the pituitary, results in testicular atrophy in dogs and rats. More evidence for hypothalamic involvement in spermatogenesis was provided by an experiment utilizing hypophysectomized rats with pituitary transplants in the kidney. A small percentage of the rats so treated showed active spermatogenesis and normal testicular weight. Implantation of testosterone into the hypothalamic median eminence of these rats produced marked testicular involution.

Receptors sensitive to decreased levels of testosterone reside in the hypothalamus (Davidson, 1967). Cyproterone, (1,2 alpha-methylene-6-chloro-delta 6-17 alpha-hydroxyprogesterone) was shown to block the action of testosterone on the reproductive system and to prevent differentiation of the male pattern of gonadotrophin secretion by neonatal androgen in the rat. Cyproterone implants in the median eminence resulted in a significant stimulation of testes, seminal vesicles, and ventral prostates of immature (thirty day Old) rats. These findings reported by Davidson suggest that an increased secretion of gonadotrophin occurs when testosterone is prevented from interacting with a receptor in the basal hypothalamic region.

Bolt (1971) reported that a single intramuscular injection of 5 mg of testosterone in oil resulted in a marked depression of plasma concentration of leutinizing hormone (LH) over a span of fifty-three hours in rams. The mean concentration of plasma LH over the fifty-three hour test period was 2.3 ng/ml for control rams compared to 0.78 ng/ml for the rams receiving a testosterone injection.

These experiments conclusively demonstrate that testosterone levels in the peripheral circulation influence the basal hypothalamic region to govern the release of releasing factors which in turn influence the outflow of gonadotrophins from the anterior pituitary gland.

Confusion exists as to the exact role of specific hormonal agents in the maintenance of spermatogenesis. However, it seems clear that gonadotrophins and testosterone are necessary for complete spermatogenesis. Testosterone has at least two target sites, the direct action on the seminiferous tubules to support spermatogenesis and on the hypothalamus of the brain to regulate releasing factor concentration in

hypophyseal-portal vessels thus regulating anterior pituitary gonadotrophin production. Thus it appears that any chemical agent interfering with gonadotrophin stimulated testosterone production by Leydig cells would result in cessation of spermatogenesis. The question remains however whether some chemical or physical agent exhibition antispermatogenic characteristics might be a more effective male contraceptive.

Factors Inhibiting Spermatogenesis

Inhibition of the spermatogenic process presents a promising approach to the problem of population control in man. However, attempts to interrupt normal body processes such as spermatogenesis are fraught with deleterious side effects.

Several specific criteria for an antispermatogenic agent must be met before it could be considered an effective systemic contraceptive for men: 1) such an agent must be one hundred percent effective; 2) the effects of the agent must be fully reversible; 3) the agent must not produce effects deleterious to the recipient or to future offspring of the recipient; and 4) there must be a simple method for chronic administration of a readily available, economically produced material.

The following sections consider immunologic reactions, temperature, irradiation, cadmium, chemicals, and steroids as inhibitors of spermatogenesis and consequently as potential male contraceptive agents.

Immunologic Inhibition of Spermatogenesis

Freund et al., (1954) provided the first demonstrations of germ cell-specific immune-induced aspermatogenesis. A mixture of tissue homogenates of testes emulsified in Freund complete adjuvant injected

into adult males of the same species (guinea pig or rat) consistently gave rise to partial or complete destruction of host germinal epithelium within six to eight weeks.

Mild reactions are evidenced by shedding of spermatozoa, spermatids and spermatocytes in few or many seminiferous tubules while severe lesions result in the desquamation of all germ cells including spermatogonia. Freund et al., emphasized that aspermatogenesis could be due to testicular lesions brought on by adjuvant administration alone.

Talaat and Laurence (1971), reported impairment of spermatogenesis after active immunization with leutinizing hormone (LH) in the adult male rat and rabbit. Bovine LH was dissolved in saline and mixed with an equal volume of complete Freund's adjuvant antigen emulsion. This was followed one week later by three intraperitoneal injections of 0.5 mg of LH in 0.5 ml saline administered at three day intervals. A pool of antisera with a high titer was obtained from actively immunized male rabbits and used in the animals for passive immunization. Active immunization of adult male rabbits and adult male rats with bovine LH resulted in an interruption of the spermatogenic process of both species as evidenced by the presence of only spermatogonia or spermatocytes in the tubules of the animals so treated thirty-five days prior to testes removal. Moreover, there was a loss of libido in both species.

Immunologic procedures, although capable of inhibiting spermatogenesis are not desirable methods. The non-specific action from allergic aspermatogenesis reported by Freund and the loss of libido following immunization with LH limit their usefulness as a male contraceptive.

Spermatogenic Inhibition by Increased Temperature

In species with scrotal testes, spermatogenesis may be affected not only by factors which affect whole body heat, but also by heat applied directly to the scrotum, insulation of the scrotum or even confinement of the testis to the body cavity as occurs in cryptorchidism. Body temperature changes were not as effective in raising the temperature of the testis as was the application of local heat according to Harrison and Harris, (1956).

The effect of heat on testicular function in mammals seems to be similar regardless of the source or means of heat application. Marked degeneration of the guinea pig testis occurs following application of water at 46 to 47 degrees centigrade to the scrotum for 15 to 30 minutes. The degeneration was complete in 12 days and the regenerative period required approximately 45 days in some tubules (Young, 1927). Bowler (1967) found that rat testes exposed to water at 43.5 degrees centigrade for twenty minutes degenerated and required 52 to 65 days to recover from a single application. One heat treatment obviously reduces the germinal epithelium to stem cell spermatogonia since the duration of spermatogenesis in the rat is 52 days (Clermont and Harvey, 1965).

Continuous high environmental temperatures (32.2 to 33.3° C) have been shown to result in low semen volume, reduced sperm mobility, decreased concentration of spermatozoa and reduced percentage of live and normal sperm in rabbits (Oloufa et al., 1951).

Findings similar to those described above in guinea pigs, rats and rabbits have been reported for dogs (Cowles, 1965), bulls (Rhynes, 1971), rams (Moore and Oslund, 1924), monkey (Venkatachalam and

Romanathan, 1962), and man (Rock and Robinson, 1965; Robinson and Rock, 1965; Robinson et al,. 1968).

Although increased temperature of the testes seems to produce spermatogenic inhibition which is universal among animals with scrotal testes such a means of spermatogenic inhibition is not desirable as a male contraceptive measure since it is extremely difficult to apply constant heating to the scrotum. Moreover, permanent injury to the germinal epithelium may result from repeated exposure of testes to heat.

Spermatogenic Inhibition by Radiation

The testis is recognized as one of the most radio-sensitive organs of the body as measured by the amount of radiation energy necessary to destroy cells of the germinal epithelial tissue. However, there are certain cell types within the testis that are radio-insensitive. These include: spermatogonia, the interstitial cells, cells comprising the tubular wall, and the Sertoli cells.

Radiation brings about infertility by maturation depletion of the cellular elements of the germinal epithelium as the intermediate and type B spermatogonia are destroyed. The sensitivity of the germinal cells to radiation is closely correlated with cellular division and the stage of individual cell development. Damage of the germinal epithelium is directly related to the amount of energy the testis is exposed to while repair is inversely related to the time after radiation (Rugh, 1960).

A recent investigation by Rugh (1971), showed that stunting was the major anomaly of offspring produced from male mice receiving 1,000 R X-rays. Irradiation is not desirable as a method of contraception

for male animals, at this time, due to the difficulty of applying the treatment, short duration of sterility induced by each irradiation treatment, and the potential hazard of genetic damage.

Spermatogenic Inhibition by Cadmium

Soluble cadmium salts have a very toxic effect on male gonads of many mammalian species. A report by Parizek and Zahor (1956) focused attention on the deleterious effects of cadmium on testicular tissue. Subcutaneous administration of a single dose (3.6 to 7.2 mg/kg body weight) of cadmium chloride brings about a rapid progression in the testes of rats and mice leading to necrosis and total destruction. World wide interest which emerged from these studies has provided more knowledge on the effects of cadmium on the testis than any other element.

Injurious effects of cadmium are often considered selective; since the administration of a dose as low as 1.8 to 3.6 mg/kg body weight provokes or induces morphological changes in rat testes without comparable changes in other organs.

The initial site of injury due to cadmium administration is not the germinal epithelium. The vasculature of the testis was shown to be the initial site of cadmium injury in several reports. Cadmium induces hemorrhagic necrosis in testes devoid of germinal epithelium such as in the testes of rats subjected to long term cryptorchidism or triethylene-melamine treatment (Gunn et al., 1963), vitamin E deficiency (Mason et al., 1964), or hypophysectomy (Gunn et al., 1963). Electron microscopy studies by Chiquoine (1964), confirmed that primary vascular lesions preceded all other changes in the parenchymal elements of testes.

Gunn and coworkers (1965) reported that following interference

of rat testes circulation, either from cadmium injury or surgical ligation (partial or complete), interstitial cell tumors developed. Moreover, these tumors form at the same rate and appear to have identical morphological characteristics.

Although cadmium may be a useful agent in the investigation of testicular function, the high degree of selective toxicity for testicular vasculature resulting in permanent damage to the testicular parenchyma and the tendency to induce neoplastic tissue makes this mineral element undesirable as a male antifertility agent.

Spermatogenic Inhibition by Chemical Agents

The chemical approach to male fertility control has received considerable attention. Excellent reviews have been prepared on the effect of drugs on spermatogenesis by Fox and Fox (1967) and Gomes (1970).

During studies of the cytostatic and cytotoxic effects of numerous chemical agents it was noted that administration of specific chemicals resulted in damaged germinal epithelium in various species of animals; nitrogen mustards produced atrophic testes in men (Spitz, 1948), and damaged spermatogonia and spermatocytes in mice (Landing et al., 1949); furadroxyl, a nitrofuran, caused extensive damage to the germinal epithelium of the rat testis (prior and Ferguson, 1950); triethylenemelamine (ethyleneimine derivative) was reported by Hendry et al., (1951) to cause spermatogenic arrest and testicular destruction in dogs and rats.

Alkylating Agents

Chemotherapeutic alkylating agents undergo chemical reactions generating highly reactive electrophilic carbonium ions that readily

form covalent linkages (alkylate) with various nucleophilic substances, including such biologically important moieties as phosphate, amino, sulfhydryl, hydroxyl, carboxyl, and imidazole groups (Calabresi and Parks, 1970). The alkylating agent's most important pharmacological actions are those that disturb the fundamental mechanisms concerned with cell growth, mitotic activity, differentiation, and function. The ability of these drugs to interfere with normal mitoses and cell division in rapidly proliferating tissues provides a basis for their inhibition of spermatogenesis.

Nitrogen Mustards. Early work by Spitz (1948) indicated that twenty-seven to thirty men treated with nitrogen mustards for therapeutic purposes demonstrated atrophic testes on postmortem. Landing and coworkers (1949) reported microscopic changes in spermatogonia and spermatocytes of mice within twenty-four hours after administration of three nitrogen mustard compounds. Jackson and coworkers (1959) and Jackson (1966), using serial mating techniques to evaluate the male antifertility effects of several nitrogen mustard compounds reported no effects except with nitrogen mustard oxide in high doses. When rats received a dose of nitrogen mustard oxide (20 mg/kg body weight) over four days, fertility was absent and mating decreased during this first week of treatment, and the rats were subfertile during the fourth week. Jackson (1961) expressed the view that, lethal or nearly lethal quantities of nitrogen mustards are necessary to significantly reduce spermatogenesis and fertility in rats.

Ethyleneimine Derivatives. Ethyleneimine derivatives are one of the principal chemical agents (among alkylating agents) that exhibit

selective interference with spermatogenesis. Triethylenemelamine given to rats in small doses (0.05 mg/kg body weight, I.P.) produced infertility even though sperm were present (Jackson and Bock, 1955). Larger doses (0.2 mg/kg of triethylenemelamine resulted in infertile rats the fourth week following a single treatment for reduced fertility prior to that time. Normal fertility was regained the fifth week after initiation of treatment (Bock and Jackson, 1957).

A variety of ethyleneimine derivatives have been examined and their effects on fertility found to be qualitatively similar. According to the dose and compound used, antifertility effects ranging from selective to complete destruction of the seminiferous epithelium can be achieved, without interference with libido (Jackson et al., 1959). Low doses of these derivatives produce sterility in rats and mice, resulting from an action on spermatids. Higher doses produce a biphasic infertility pattern, including an antispermatogonial effect associated with aspermia.

Following a single intraperitoneal injection (10 mg/kg body weight) of 1, 4-dimethanesulfonoxybutane (busulfan, Myleran), male rats remained fertile for seven to eight weeks, but became completely infertile during week eight to nine. Infertility persisted for one to four additional weeks depending on the dose administered (Jackson, 1959).

The action of straight-chain monoester of methanesulfonic acid is confined to spermiogenic cells, whereas the isopropyl ester affects all premeiotic cells. Diesters have a wide range of antifertility action in the rat with the spermatogenic cell stage affected varying with the compound used (WHO Bulletin Number 424, 1969).

All the chemotherapeutic alkylating agents may affect DNA molecules

of cells to cause miscoding with ultimate substitution of an adenine-thymine base pair for a guanine-cytosine base pair, opening of the imidazol ring or depurination by excision of guanine residues and cross-linking of two nucleic acid chains. Any of the above effects could produce cytotoxic or mutagenic effects. The toxicity or mutogenic effects of alkylating agents make them undesirable for use as male antifertility agents in human populations.

Nitrofurans

The nitrofurans are a class of aromatic nitro compounds which have proven effective as cytostatic agents (Prior and Ferguson, 1950). The feeding of 200 mg/kg body weight of furadroxyl per day to rats resulted in extensive damage to the germinal epithelium of the testis. The degenerative changes began four to eight days after onset of drug administration. At the end of one week, the sperm, spermatids, and secondary spermatocytes were absent or exhibited abnormal morphology. Regenerative changes were observed and normal appearing tubules were present four weeks later (Prior and Ferguson, 1950).

Investigations by Nelson and Steinberger (1952) confirmed the results reported by Prior and Ferguson. These investigators stated that spermatogenic arrest occurred in rats fed 1.5 g per kg. diet for 30 days at the primary spermatocyte stage while body weight, growth, and androgen production were impaired only slightly.

Nelson and Steinberger (1953) reported the effects of three nitrofurans (furacin, furadroxyl and furadantin) upon male reproduction. The minimum effective levels per kilogram of diet in animals thirty to thirty-five days of age, treated until sixty days of age were: furacin

0.2 g, furadroxyl 0.75 g and furadantin 1.5 g. That testicular damage is not permanent was shown by the fact that animals maintained on the drug for one hundred days completely recovered as manifested by fertile matings after sixty to one hundred twenty days.

Paul et al., (1954) reported that furacin inhibited the formation of acetyl coenzyme A from pyruvate. This action by a nitrofuran could account for the body weight depressing action of the high doses necessary to inhibit spermatogenesis. Such side effects prohibit the use of nitrofurans as antifertility agents.

Bis(dichloroacetyl)diamines

The bis(dichloracetyl)diamines that have been tested have been found to suppress spermatogonia, spermatocytes and spermatids without affecting gonadotrophin secretion in rats, mice, guinea pigs, dogs, and monkeys (Coulston et al., 1960), (Drobeck and Coulston, 1962).

Heller and coworkers (1961) tested two of these compounds, N, N' (p-phenylenedimethylene) bis(2, 2-dichloro-N-ethylacetamide) in man. Courses of treatment caused a gradual fall in sperm counts to a very low level in eight to ten weeks. Their slow action and incomplete inhibition of spermatogenesis limits the practical application of these chemicals as male antifertility agents.

Spermatogenic Inhibiton by Gonadal Hormones

The ability of steroidal hormones from the gonads to inhibit spermatogenesis bears close scrutiny. This antispermatogenic function probably is derived from the feed-back of such hormones on the hypothalamus to control pituitary gonadotrophin release.

Estrogenic Inhibition of Spermatogenesis: Severe damage has been

observed in testes of rats (Ludwig, 1950); (Steinberger and Nelson, 1955); men (Heckel and Steinmetz, 1941); bulls (Cupps, 1960); boars (Wallace, 1949) and hamsters (Bacon and Kirkman, 1955), following estrogen administration. The suppression of spermatogenesis by estrogens is considered to be a secondary response resulting from suppression of circulating gonadotrophin levels (Ludwig, 1950); (Steinberger and Nelson, 1955).

Ludwig (1950) administered estradiol (8.4 µg/day) to thirty day old male rats until they reached sixty days of age. This level of estrogen produced germinal epithelial inhibition and atrophy of testicular interstitial tissue that was evident on histological examination of the testes at sixty days of age. The inhibition of the spermatogenic process was comparable to that found in hypophysectomized rats, with spermatogonia and primary spermatocytes the only stages present in seminiferous tubules. Steinberger and Duckett (1967) administered estradiol benzoate in graded doses to rats: one to ten days - 10 µg; ten to twenty days - 20 µg; twenty to forty days - 30 µg; forty to sixty days - 40 µg. Their results confirmed those of Ludwig in that the seminiferous tubules were markedly reduced in size and that inhibition of the spermatogenic process occurred at the primary spermatocyte stage.

Testicular degeneration has been reported in man following treatment with stilbesterol (Dunn, 1941) and other estrogens, (de la Balze et al., 1954). However, the potential of estrogens to produce feminization in the male precludes their consideration as a male antifertility agent.

Progestational Inhibition of Spermatogenesis. Many studies have

been reported regarding the effect of progesterone or progesterone-like drugs on female fertility. However, there is a paucity of information about the effect of progestins on male animals.

Heller et al., (1959) reported the effects of progesterone and synthetic progestins upon the reproductive physiology of normal men. Following four weeks of daily intramuscular injections of 50 mg progesterone four of five men were azoospermic. Libido and testicular size were reduced and gynecomastia occurred in two subjects. Such side effects raise serious objections to the use of progesterone as a male antifertility agent.

The synthetic progestins, (Norlutin, Enovid, and ethynyle-estradiol-3-methyl ether) seem to reduce spermatogenesis via gonadotrophic hormone depression (Heller et al., 1959). Another synthetic progestin, Nilevar depressed spermatogenesis and decreased gonadotrophic hormones without inducing estrogenic actions. Such observations led to further studies of the action of progestational agents.

Cyproterone acetate (6-chloro-60 dehydro-17 alpha acetoxyl-1, 2 alpha-methylene progesterone) is the most potent progestin yet encountered (250 times as active as progesterone when administered by injection). This synthetic steroid, although a potent progestin has received more attention as an anti-androgenic substance. Cyproterone acetate is a competitive inhibitor of the stimulatory effects of androgens including the maintenance of weight and normal histology of the seminal vesicles and the ventral prostate (Neumann et al., 1966) (Goldfoot et al., 1971) (Walsh and Gittes, 1970).

The contraceptive properties of cyproterone acetate have been demonstrated in rats (Whalen and Luttge, 1969) (Prasad et al., 1970).

Edwards (1970) reported the androgen dependent aggressive behavior of mice was unaffected by cyproterone acetate while exerting strong anti-androgenic effects on the seminal vesicles. Although the anti-fertility properties of this synthetic steroidial anti-androgen appear to be excellent in laboratory animals such as rats, mice, and guinea pigs the effect in man is vague. Rausch-Stroomann et al., (1970) reported that 100 and 200 milligram doses daily for nine months exhibited no effect on spermatogenesis in adult men.

Further work with cyproterone in the human may clarify the confusing results reported to date. In light of the inconsistent results the prospect for the use of this synthetic steroid as an antifertility agent are not as good as for the use of a natural steroid.

Testosterone Inhibition of Spermatogenesis. It has been demonstrated that testosterone decreases the peripheral blood levels of follicle stimulating hormone and interstitial cell stimulating hormone in male mammals. Ludwig (1950) reported that 60 day old rats given 0.1 mg testosterone propionate daily for 30 days resulted in a decreased gonadotrophic potency of their pituitary glands to only 31 percent of the control animals. She administered varying doses (0.1 mg, 1.0 mg, and 3.0 mg) of testosterone propionate to 60 day old rats, daily for 30 days. Using ovarian weight increases of immature female rats following injection of a suspension of pituitaries from the treated and control rats she reported gonadotrophic potency decreases of 69, 87 and 88 percent respectively for the three treatment groups.

Steinberger and Duckett (1968) reported a drop in pituitary FSH levels and an elevation of plasma FSH levels shortly after castration of male rats. Administration of testosterone to castrated male rats

prevented the drop in pituitary FSH levels and rendered blood levels undetectable. These results support the hypothesis that testosterone has an inhibitory effect on the release of FSH from the pituitary gland.

Watanabe and McCann (1969) reported a decline in hypothalamic follicle stimulating hormone releasing factor, following castration of adult male mice, which was apparent within 8 and persisting for at least 29 days postoperatively. When intact rats were treated with a large dose of testosterone propionate (2 mg/day) for 2 weeks, there was no consistent alteration in the level of stored follicle stimulating hormone releasing factor. These results are consistent with the view that increased testosterone levels in the peripheral blood inhibit the release of follicle stimulating hormone.

Pelletier (1970) reported that a single intramuscular injection of 400 milligrams of testosterone propionate into a castrated ram decreased the plasma leutinizing hormone level significantly by the first day after treatment. Moreover, the plasma leutinizing hormone levels remained at a very low level for 5 to 7 days, returning to normal after day 9. These reports suggest that testosterone blocks the discharge of leutinizing hormone.

The inhibiting effects of the male sex hormone on testis weight and spermatogenesis was reported by Ludwig (1950). She found that testis weight of rats receiving doses of 0.1 mg of testosterone propionate (TP) daily were reduced 25 percent from control testis weights. In contrast testis weights of rats receiving 1.0 and 3.0 mg TP/day were reduced only 17 and 8 percent respectively. Histological evidence showed that inhibition of spermatogenesis paralled the loss of

testicular weight. Thus, the above results suggest that low dosages of TP decrease gonadotrophin release from the anterior pituitary but are insufficient to maintain the seminiferous epithelium.

Heller and coworkers (1950) tested the effect of exogenous testosterone in men. Thirteen men received 25 milligrams testosterone propionate daily for 24 to 29 days. Biopsies taken at the cessation of the treatment indicated that damage included sclerosis and hyalinization of the membranes of the seminiferous tubules, atrophy and necrosis of germinal elements, disappearance of the Leydig cells and arrest of sperm formation. Biopsies, performed 5 to 6 months after treatment was stopped, indicated partial recovery of the testicular parenchyma. Subsequent biopsies taken, up to 31 months after stopping treatment, revealed marked improvement over the biopsies taken at the end of the period of testosterone administration. This experiment proves two important facts: first, azoospermia can be produced in man by the administration of testosterone and second that the azoospermia is reversible.

From studies reported by Woods and Simpson (1961), maintenance of accessory sex organ weight was accomplished with doses of testosterone propionate much lower than doses that would stimulate spermatogenesis in hypophysectomized rats. Therefore, since low doses of testosterone inhibit the release of pituitary gonadotrophins but do not maintain spermatogenesis it may be possible to administer chronic low dosages of testosterone which will maintain the peripheral blood level of the hormonal steroid at normal levels, maintain libido and accessory sex organs of an intact animal while inhibiting spermatogenesis. Thus the critical problem becomes one of finding the best method of administering testosterone over a long period of time at a constant rate to an animal.

Silicone Rubber as a Carrier for Prolonged Drug Therapy

While performing *in vitro* studies on artificial heart valves, Folkman *et al.*, (1964) noted that silicone rubber (Silastic) possessed the property of absorbing certain dyes from solution and subsequently giving off these dyes. This observation suggested that silicone impregnated implants might serve as a mode of administering various pharmacological agents to man over prolonged periods of time.

A substance which can act as an intermediary between an animal's body and foreign materials to be administered to the animal should have certain characteristics. Scales (1953) defined properties necessary for an ideal soft tissue substitute: 1) not physically modified by soft tissue; 2) chemically inert; 3) does not cause inflammation or foreign body reaction; 4) noncarcinogenic; 5) produces no state of allergy or hypersensitivity; 6) capable of resisting mechanical strains; 7) capable of fabrication in the form desired; and 8) capable of sterilization. The silicones have been found to have these properties to a degree sufficient to make them very useful for long-time subdermal contact in an animal's body (Braley, 1965).

Steroid Diffusion Through Silicone Rubber Membranes

Dzuik and Cook (1966) placed steroids in silicone rubber capsules which were then incubated in saline solution at 37.5° C. Estradiol, progesterone, androgestenedione, testosterone, cortisone, 6 alpha-methyl-17 alpha-acetoxy-progestrone, and 17-acetoxy-6-methyl-16-methylenepregna-4, 6 diene-3, 20-dione were all found to pass through the capsules into saline solution at quite constant rates for several days. Capsules containing one of the steroids, megesterol acetate,

were implanted subcutaneously in normal cycling ewes. The incidence of estrus was reduced during the 34 day period while the implants were in place. Estrus was resumed upon removal of the implants.

Further evidence of steroid diffusion through polydimethylsiloxane (PDS) membranes was provided by Kincl et al., (1968). This research group conducted experiments on the diffusion of several steroids through PDS membranes into distilled water. Testosterone was found to diffuse out of the PDS capsules into distilled water at a rate of 2.6 μg/hr per 100 mm^2 surface area and wall thickness of 0.508 mm. Moon and Bunge (1968) showed that enough testosterone was released through the wall of PDS capsules over a 16 week period to maintain the accessory sex glands of mature castrate male rats.

That continuous dosage of steroids via PDS capsules implanted subcutaneously enhances the biological effect of steroids in rats and rabbits was shown by comparing subcutaneous injection, gavage, and PDS routes of administration (Chang and Kincl, 1968). From 6 to 25 times less megesterol acetate was needed via the PDS implants to produce comparable biological effects. The same workers (Chang and Kincl, 1970) reported that testosterone-filled PDS capsules were thirty times more active than an oil solution of testosterone in the parabiotic rat assay and about fifteen times more active in androgenic test in castrated adult male rats.

Silicone rubber exhibits properties which are attractive for chronic administration of testosterone. However, the release rate over prolonged time periods needs more investigation and the release rate needed to effect spermatogenic inhibition without concommittant accessory sex organ hypertrophy must be elucidated.

Summary

Research in fertility control in the male has been carried out largely in laboratory animals using both chemical and immunological approaches. Immunological processes present hazards when used in man and they suffer from a lack of specificity. Similarly, none of the chemical agents described in the preceding sections are suitable for use in man due to slow action, incomplete inhibition of spermatogenesis, mutogenicity, and known or potential toxicity.

The preceding review demonstrates that spermatogenesis depends on gonadotrophin stimulated testosterone secretion by the interstitial cells of Leydig. The local concentration of testosterone required to maintain spermatogenesis is greater than the peripheral level of testosterone necessary for maintenance of accessory sex organ function and brain stimulation. Perhaps the development of techniques that would permit testosterone to be administered at constant rates for prolonged periods would be a valuable procedure to inhibit spermatogenesis without producing undesirable side effects.

The relationship between PDS capsule dimension and testosterone release rate, the effect of a specific dosage of testosterone upon spermatogenesis, libido and accessory sex organ function in male rabbits must be established to test the hypothesis that a continuous administration of testosterone via the appropriate sized PDS capsules implanted into male rabbits will inhibit spermatogenesis without producing accessory sex organ hypertrophy.

CHAPTER III

MATERIALS AND METHODS

Materials

Silicone Rubber

Polydimethylsiloxane capsules were prepared from Silastic Medical-Grade Tubing purchased from Dow-Corning Corporation through the Midwest Surgical Supply, Oklahoma City, Oklahoma. Two sizes of tubing were used in this study; 1) catalogue number 602-331, inside diameter 3.35 millimeters, outside diameter 4.65 millimeters and wall thickness 0.65 millimeters, 2) catalogue number 602-331, inside diameter 1.47 millimeters, outside diameter 1.96 millimeters and wall thickness 0.245 millimeters.

Dow-Corning Medical Adhesive Silicone Type A was used to seal the ends of the various lengths of silicone rubber tubing. This particular adhesive cures to a firm, non-sticky consistency within 24 hours in a water vapor atmosphere.

Animals

Mature male New Zealand White Rabbits were acquired from the Redwood Game Farms, Salt Lake City, Utah.

Steroids

Testosterone (17 beta-hydroxyandrost-4-ene-3-one) was obtained from Sigma Chemical Company and cholesterol (5-cholestin-3-ol) from

Steraloids, Inc., Pawling, New York.

Radioactive Isotopes

Testosterone-1,2-^{3}H was obtained from New England Nuclear Corporation, Boston, Massachusettes and was purified via thin-layer chromatography. Purity was checked by a radio-chromatogram scanner (Packard, Model 7201) and identity confirmed by the use of authentic testosterone standard in adjacent lanes on the thin layer plates.

Tritiated toluene, 2.28×10^{6} dpm/g, was obtained from Packard Instrument Company, Inc., Downers Grove, Illinois and used as tritium standard in the calculation of quench correction curves.

Scintillation Counting Material

Two scintillation fluids were used in this study. 1) Aquasol purchased from New England Nuclear, Boston, Massachusetts; and 2) toluene scintillation fluid containing 12 g of 2,5-diphenyloxazole (POP) and 0.12 g of 1,4-bis-1-(5 phenoxazolyl)-benzene (POPOP) in 3 liters spectroquality toluene. The latter mixture was allowed to equilibrate at least 24 hours before use and stored in the dark.

Reagents

The nanograde solvents obtained from Mallindkrodt Chemical Works included dichloromethane, ether, benzene, ethyl acetate, toluene and methanol. Water used in the procedures was distilled two times over glass, ether-washed and redistilled.

Silica Gel

Silica gel (Silica TLC-7GF, Mallinckrodt) was washed three times with dilute acetic acid over a scintered glass filter, followed by

three rinses in boiling triple distilled water and washed two times with boiling methanol. The silica gel was dried 24 hours at 110° C. Thin layer chromatography plates were poured (0.25 mm thick) with slurry containing 33 g silica gel to 78 ml water.

Glassware

Immediately after use glassware was rinsed, soaked in detergent, brushed and placed in chromic acid overnight. Following seven to eight tap water rinses, the glassware was soaked in detergent, rinsed again with tap water and placed in dilute hydrochloric acid. The acid bath was followed with rinses as follows: ten times with tap water, ten times with distilled water and two times with nanograde methanol. The glassware was subsequently air dried at room temperature.

Solution for Tissue Homogenization

The solution used for homogenization of testicular and epididymal tissues was prepared by mixing 9 g NaCl, 0.5 ml Triton X-100, and 0.1 ml of 1.0 g/ml Methiolate solution in 1 liter of glass distilled water. Triton X-100 was added to prevent fat emulsification which sometimes obscures cells and methiolate to retard bacterial proliferation (Amann and Lambiase, 1969).

Tissue Fixative Solutions

Zenker-formol fixative solution was used for testicular tissue. Zenker stock base is prepared 24 hours before use as follows: 50 g mercuric chloride, 25 g potassium dichromate, and 10 g sodium sulfate are mixed into sufficient distilled water to make 1,000 milliliters of solution. Immediately prior to the addition of testicular tissue 5 ml of 40 percent formaldehyde is added to fix one rabbit testis.

Methods

Recrystallizztion of Steroids

Testosterone and cholesterol, were purified via recrystallization (Axelrod et al., 1965).

Testosterone. One gram of testosterone (Sigma Chemicals) was placed in a 35 ml conical vial. Methanol (nanograde, Mallinckrodt) was added with a pasteur pipette while the vial was suspended in water at 70 to 80° C. A minimal quantity of methanol was added to dissolve the testosterone. The vial was removed from the water bath, held at room temperature for four hours, followed by four hours at 5° C then twelve hours at -20° C. The methanol solution was removed, the crystals dissolved in fresh methanol and recrystallized again. The melting point was determined after the second recrystallization (Thomas-Hoover Capillary Melting Point Apparatus) and recrystallization repeated until a constant melting point was obtained (153° C). The crystals were placed in a vacuum jar containing phosphorus pentoxide and dried for 72 hours.

Cholesterol. The same method of recrystallization was used for cholesterol as for testosterone with the exception that methanol: ethanol (1:1 v/v) was used for the solvent and that the melting point was 148° C. Although this method will not provide cholesterol of high purity it was sufficient for the purpose of this investigation.

Check of Identity and Purity of Testosterone-1,2-^{3}H

Thin layer chromatography was used to check the identity and purity of testosterone-1,2-^{3}H (New England Nuclear). A 50 microliter

aliquot of testosterone-1,2-^{3}H (New England Nuclear). A 50 microliter aliquot of testosterone-1,2-^{3}H was spotted on a thin layer plate. Authentic testosterone was placed on an adjacent lane of the same plate. The plate was developed in benzene: ethyl acetate, 55:50 (v/v), air dried and the R_f of authentic testosterone determined visually with UV light. The R_f of the testosterone-1,2-^{3}H was determined by subjecting the thin layer plate to a radio chromatogram scan. The identical R_f of the testosterone-1,2-^{3}H and the unlabeled testosterone was accepted as proof that testosterone-1,2-^{3}H was essentially pure.

Preparation of Tritium Labeled Testosterone

Purified testosterone-1,2-^{3}H (thin layer chromatography) of known specific activity and known volume was added to a 35 ml conical tube then dried under a stream of nitrogen. A known amount of testosterone recrystallized to a constant melting point was added to the vial. The crystals were dissolved in a minimum amount of hot methanol and cooled slowly as described in the testosterone recrystallization method. After crystal formation occurred the mother liquor was placed in a water bath (60° C) until the vial was warm to the touch and glass distilled water added drop-wise until a white cloud-like precipitate lingered in solution. The solution was removed from the water bath and slowly cooled as previously described. Approximately 95 percent of the original mass of testosterone crystals were recovered by repeating the above procedure several times. The crystals were dried as described previously.

Specific Activity of Testosterone-1,2-^{3}H Used to Pack PDS Capsules

The specific activity was determined by determining the mass of

a single crystal via a Cahn Electronic Balance and measuring the radioactivity of that crystal utilizing a Packard liquid scintillation spectrometer, Model 3003.

Polydimethylsiloxane Capsule Preparation

Silastic Medical Grade (Dow-Corning Corporation) silicone rubber tubing was used to prepare all the capsules. Tubing with inside and outside diameters of 3.35 mm and 4.65 mm respectively and thickness of 0.65 was selected for Experiments 1, 2, and 3. Tubing with inside and outside diameters of 1.47 mm and 1.96 mm, (0.245 mm thickness) respectively was selected for Experiments 4 and 5.

The tubing was cleaned by washing in a hot water-soap solution to remove sodium bicarbonate (dusted on surface to facilitate handling) and other surface contaminates. Ivory Flakes was used since oily soaps or synthetic detergents are contraindicated due to the property of Silastic to absorb the latter materials. The tubing was rinsed with hot tap water, distilled water, and air dried. The desired length of tubing was cut, one end sealed with 3 mm of Dow's Medical Adhesive Silicone Type A and allowed to cure at room temperature and atmosphere for 24 hours. PDS tubing with one end sealed and cured was filled to the desired length with testosterone-tritium (SA = 1900 cpm/ug) for Experiments 1, 2, and 3 and unlabeled testosterone or cholesterol for Experiment 4 and 5. The open end was sealed with Medical Adhesive Silicone Type A and cured as previously described.

Protocol for Calculation of PDS Capsule Surface Area. Studies from several laboratories have shown that steroids diffuse through PDS at rates proportional to membrane area and inversely proportional

to membrane thickness (Garrett and Chemburkar, 1968; Kincl et al., 1968; Kratochivl et al., 1970).

The surface area of a cylinder equals the circumference times the length. The circumference of a cylinder equals two times pi times the radius of the cylinder; hence the surface area of a cylinder equals two times pi times the radius times the length of the cylinder. The external surface area (area of the membrane in contact with body fluids when implanted) of the PDS capsules is reported in this study. The circumference was calculated from the outside diameter of the Silastic tubing as given in the specifications from the Dow-Corning Corporation. The length of tubing used in calculation of surface area was the actual length of tubing filled with testosterone.

Scintillation Counting

Water Sample Preparation. One milliliter aliquots of water bathing the testosterone-tritium filled PDS capsules (Experiment 1) were added to 14 ml of Aquasol (New England Nuclear), shaken, cooled at 5° C for four hours then counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003, using the Automatic External Standard to determine the percent efficiency of the counting procedure. The mean of three ten minute counts minus background was used for calculation of the tritium concentration in the water.

Plasma Sample Preparation. Plasma samples from Experiments 2 and 3 were thawed with a stream of warm air, 1 ml pipetted into 14 ml of Aquasol, mixed well by vigorous shaking and allowed to equilibrate 48 hours at 5° C prior to counting. The equilibration time was necessary with plasma samples to obtain consistent counts from each vial. The

mean of three ten minute counts minus background was used for calculation of tritium concentration in the plasma.

Quench Correction. The object of this procedure was to utilize the Automatic External Standard (137 Cs) device of a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003, to determine the individual counting efficiency for each sample.

A rectangular coordinate system was used to construct a quench curve from a one-to-one correspondence between sets of points in a plane. Percent of counting efficiency was designated as points on the ordinate (y axis) and counts per minute in the Automatic External Standard (blue channel) was designated as points on the abscissa (x axis). Such a curve must be prepared according to a given set of specifications: 1) type of scintillation fluid, 2) standard source of radioisotope or isotopes, 3) quenching agent used, and 4) liquid scintillation spectrometer settings adjusted for single label or double label counting.

For this study a quench curve for tritium in Aquasol scintillation fluid was desired. Nitromethane was used as a quenching agent, tritiated toluene as a source of radio-activity, and the measurements were made with single label settings on the liquid scintillation spectrometer.

The following materials were added to 18 scintillation vials: 14.5 ml of Aquasol to all vials, 0.1 ml tritiated toluene to 16 vials, and nitromethane in graduated amounts (0.0005, 0.01, 0.05, 0.08, 0.10, 0.20, and 0.40 milliliters) to duplicate vials (14 total vials).

Thus two vials contained only Aquasol to provide background measurements, two vials contained no quenching agent to provide radioactivity measurement of the tritium used as a standard and fourteen

vials contained tritiated toluene plus graduated amounts of quenching agent. Each sample was counted for three periods of ten minutes each.

The radioactivity of the tritium standard equaled 2.28×10^6 dpm/g, which was in a 10 ml volume of toluene. The density of toluene equals 0.8669 g/ml. The 0.1 ml used in each scintillation vial represents (2.28×10^6 dpm/g times 8.669×10^{-2} g/0.1 ml) 1.977×10^5 dpm/0.1 ml of tritiated toluene.

To determine the efficiency of the counting apparatus the following formula was used.

$$\text{Counting efficiency} = \frac{\text{counts/minute actually determined}}{\text{counts/minute expected from known activity}}$$

The quench curve constructed with data from the above procedure (counting efficiency versus blue channel AES cpm) was then used for correcting the measurement of radioactivity in each sample from Experiments 1, 2 and 3.

In Vitro Release of Testosterone from PDS Capsules

Testosterone-tritium filled PDS capsules with the ends properly cured were rinsed with methanol, dried and placed in Erlenmeyer flasks containing 100 ml distilled water. One millimeter samples were withdrawn at 6, 12 and 24 hours. The 1 ml of bathing media was added to 14 ml Aquasol scintillation fluid, equilibrated in the dark at 5° C for 1 hour then counted in a Packard Liquid Scintillation Spectrometer, Model 3003. To identify the tritiated material in the water bathing the testosterone-tritium filled PDS capsules, 100 ml of the incubation water was extracted three times with dichloromethane, the organic extract separated, dried under a stream of nitrogen and concentrated in the tip of the conical tube with methanol. The residue in the tube

was put into solution with 1 ml of methanol, one-half milliliter was transferred to scintillation vial, dried under nitrogen, toluene scintillation fluid (POP, POPOP) added and counted on a liquid scintillation spectrometer (Packard, MOdel 3003). The other one half milliliter of the extract was transferred to a TLC plate, testosterone standard spotted on the adjacent lane and developed in benzene:methanol (87.5: 12.5 v/v). A chromatogram scan (Packard, Model 7201) was performed. The R_f value of authentic testosterone and the extract of the water bathing the capsules was compared to identify the material in the extract.

Animal Housing and Preparation

The rabbits used in this study were housed individually in an air-conditioned room on a 14:10 hour light-dark schedule and provided with Purina Rabbit Chow and water ad libitum.

Three weeks accommodation time was allowed for the rabbits after arrival. They were treated for ear mites, teeth and toe nail problems during this time.

Castrations and subcutaneous and intraperitoneal capsule placement (Experiments 2 and 3) were performed with the rabbits under pentobarbital sodium anesthesia. Subcutaneous implantation of capsules in experiment 4 was made using a 12 gauge cannula and a 15 gauge stylet without anesthetic.

A bleeding technique described by Hoppe, et al., (1969) was used to facilitate obtaining blood samples and avoid the mortality risk associated with repeated cardiac puncture. The blood samples for experiments two, three, and four were collected into oxylated tubes, and stored in ice until the samples could be centrifuged to obtain the blood

plasma. The plasma was frozen at -5° C until analyses were performed.

Determination of Plasma Testosterone Concentration

Testosterone was extracted, isolated and quantified via gas-liquid chromatography equipped with electron capture detection as originally described by Brownie, et al, 1964, and modified by Kirschner and Coffman, 1968.

Preparation of Plasma for Solvent Extraction. Fifty microliters of tritium labeled testosterone standard (1,500 dpm of testosterone-1,2-^{3}H) was pipetted into 80 ml extraction tubes and evaporated to dryness under a stream of nitrogen. Plasma samples from Experiment 4 were thawed and 15 ml added to the extraction tubes containing labeled standard. The tube was then gently vortexed and allowed to equilibrate for 30 minutes at 5° C.

Extraction and Alkali Washing. The plasma was extracted three times with 45 ml of ice-cold dichloromethane. The extract was evaporated under nitrogen and the residue taken up in 30 ml of ethyl ether.

Alkali wash was accomplished by adding 10 ml of 0.1 N sodium hydroxide solution to the ether extract and gently inverting the tube twenty times. After removing the alkali phase, the organic phase was washed three times with 15 ml of water and evaporated to dryness under nitrogen.

Chromatographic Isolation of Testosterone. Thin layer chromatography plates were marked into nine lanes, eight for samples, and one for standard testosterone. The samples were spotted in benzene and developed in benzene: ethyl acetate (55:50, v/v). Since samples did

not contain sufficient testosterone to visibly absorb ultra-violet light, areas corresponding to the standard testosterone were eluted. In this solvent system testosterone had an R_f of 0.3.

Appropriate areas of silica gel containing testosterone were scraped from the TLC plate and placed in 35 ml conical tubes. This silica gel was extracted three times with a benzene:water partition system, using 1 ml of benzene with ten drops of water. The benzene fraction was pooled in 12 ml conical centrifuge tubes and evaporated to dryness under nitrogen.

Formation of the Heptafluorobutyrate Derivative. One hundredth milliliter of a 2% solution of heptafluorobutyric anhydride in hexane and tetra-hydrofuran was added to the sample tube. The tube was sealed and incubated in a 60° C water bath for thirty minutes. After refluxing, the sample was evaporated to dryness.

Chromatographic Isolation of Testosterone Heptafluorobutyrate (THFB). Following concentration by benzene, the residue was spotted on a TLC plate, with benzene, and developed in benzene: ethyl acetate (87.5:12.5 v/v). In this solvent system THFB had an approximate R_f of 0.5. The silica gel in the area co-chromatographing with the THFB standard was placed in a 35 ml tube and extracted three times with a benzene: water partition system. These extractions were carried out, using 1 ml benzene with 10 drops water. The benzene was pooled and dried under nitrogen. Following the addition of 1 ml benzene, a 0.2 ml aliquot was taken for scintillation counting, a 0.5 ml aliquot for gas-liquid chromatography.

Gas-Liquid Chromatography. A Selectra Series 5000 Barber-Coleman gas chromatograph equipped with a Model 5120 electron capture detector was used to measure the heptafluorobutyrate derivative of testosterone. Samples were applied in 5 to 10 µl of benzene. A U-shaped glass column 91.5 cm long, with 4 mm internal diameter), silanized with a 5% solution of dimethyldichlorosilane in toluene and packed with Gas Chrom Q (80 to 100 mesh) coated with 3% OV-225 (Applied Science Laboratories, Inc.), was used to effect separation of steroids. Column bath temperature was 200° C. Pure, extra dry nitrogen was used as a carrier gas with an outlet velocity of approximately 100 ml/minute.

The electron capture detector was operated in the DC mode and voltage was adjusted to give a standing current 60% of that obtained at the plateau of the voltage input curve. The detector bath temperature was 240° C. Sensitivity and linearity of detector response to standard HFB derivatives of testosterone and 20β-OH progesterone were ascertained each day of data collection on GLC. Samples were not run unless the response was linear over the range of expected THFB concentration. The size of peaks was calculated as follows: $\frac{\text{Base x Height}}{2} = \text{Area}$

Monitoring Testosterone Loss Through the Method. Testosterone loss prior to GLC was determined by measuring the loss of testosterone-1,2-^{3}H added to the plasma sample before extraction. After radioactive samples were placed in scintillation vials, they were evaporated to dryness under nitrogen, and 12 ml of scintillation fluid (POP, POPOP), was added to each vial. The samples were counted in a three channel Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003, equipped with a ^{137}Cs Automatic External Standard device. Adjustments for

losses incurred during quantitation by GLC were made by the addition of an internal standard (25 nanograms 20β-OH progesterone HFB per sample) as outlined by Horning et al., (1963).

Calculation of Testosterone Concentration. The amount of testosterone in 1 ml of plasma was calculated according to a formula used by Stabenfeldt (1968). The formula used is as follows:

Testosterone (ng/ml) = R x C x U x A x 0.596 x Df x X x P

Where:

$$R = \frac{\text{cpm testosterone-1,2-}^{3}\text{H added to the plasma}}{5 \times \text{cpm in 20\% aliquot obtained prior to GLC}}$$

$$C = \frac{\text{peak area (cm}^2\text{) 10 ng 20β-OH progesterone HFB standard}}{\text{peak area (cm}^2\text{) 10 ng THFB standard}}$$

$$U = \frac{\text{peak area (cm}^2\text{) THFB in sample}}{\text{peak area (cm}^2\text{) 20β-OH progerterone HFB in sample}}$$

A = ng 20β-OH progesterone HFB added as internal standard

$$0.596 = \frac{\text{molecular weight of testosterone}}{\text{molecular weight of THFB}}$$

$$Df = \frac{\text{(100-hematocrit) (blood volume)}}{\text{[(100-hematocrit) (blood volume)] - milliliter anticoagulant}}$$

$$X = \frac{1}{\text{GLC aliquot}}$$

$$P = \frac{1}{\text{milliliters plasma extracted}}$$

Sexual Behavior and Ejaculation

A determination of sexual behavior was performed on all the animals of experiment four. Sex behavior was measured over a 20 day period, (April 15 to May 5, 1971) by taking two ejaculates per day on alternate days. This provided a total of twenty ejaculates per animal. Teaser does were placed in a buck's cage and sex behavior scored as follows:

Point spread 0 to 10. Ten for rabbit ejaculating 0 and 60 seconds with routine female; nine for ejaculates between 60 and 180 seconds with routine female; eight for ejaculates between 0 and 60 seconds with new female; seven for ejaculates between 60 and 180 seconds with new female; six when buck mounts and thrusts vigorously with both females but does not ejaculate; five for mounting and thrusting vigorously only with the second or new female; four for mounting within 0 to 30 seconds on routine female but failing to thrust vigorously; three for mounting within 30 to 180 seconds on routine female but failing to thrust vigorously; two for mounting between 0 to 30 seconds on second female but failing to thrust vigorously; one for mounting between 30 and 180 seconds on second female but failing to thrust vigorously; 0 for rabbits failing to mount either female, (MacMillan et al., 1969). The ejaculate was collected in a graduated collection tube and placed on ice as soon as possible. The seminal volume was determined then the sample was centrifuged in a Sorval at 5° for 10 minutes to sediment any cellular material.

Analysis of Citric Acid in Seminal Plasma

Citric acid in the rabbit seminal plasma obtained in experiment four was quantified spectrophotometrically according to the method of Saffran et al., (1948).

Analysis of Fructose in Seminal Plasma

Fructose in the rabbit seminal plasma obtained in experiment four was quantified spectrophotometrically by the method of David et al., (1967).

Quantitation of Gonadal Sperm Cells

In experiment four, the testes and epididymidies were removed from each rabbit and placed on a wet paper towel. The testis was dissected from the epididymis and the epididymis dissected into head-body and tail. Individual weights were then obtained for thhe paired testes, head-body and tail of the epididymidies. Approximately one-half of one testis was weighed, tunics removed and the remaining parenchyma minced with scissors and homogenized in the micro-attachment of a Waring Blender. Similarly the dissected parts of the epididymidies were weighed, minced and homogenized.

The method described by Amman and Lambiase (1969) wase used: One half of the testis parenchyma was minced and ground in a Waring Blender for 1.5 minutes with 50 ml of SMT solution, placed in a 600 ml beaker, allowed to stand for two to three minutes, mixed by rotation of the beaker and a 10 ml sample pipetted into a 50 ml Erlenmeyer flask. Quantitation of spermatids and spermatozoa was accomplished by shaking the flask thirty times, removing aliquot with a Pasteur pipette and placing aliquot on a hemacytometer chamber. Counting was begun after the suspension had settled for approximately three minutes. Two observers made duplicate counts of each sample.

Calculation for Converting Hemacytometer Counts to Sperm Cell Counts. Calculations are based on the fact that counting is carried out without further dilution after homogenization. These calculations are all based on a 5 square count or a count involving 80 small squares of a hemacytometer. The following equations were used:

$$\text{Number of sperm cells} = \frac{(\text{number of cells counted})\ (1000)\ (4000)}{\text{number of small squares (80) counted}}$$

The above equation can be reduced to:

Number of sperm cells = (number of cells counted) (50,000)

$$\text{Total number of cells per unit of tissue homogenized} = \frac{\text{(number of cells counted) (50,000)}}{\text{(volume of homogenate)}}$$

$$\text{Number of cells per gram of tissue} = \frac{\text{number of cells/unit of tissue}}{\text{grams of tissue homogenized}}$$

$$\text{Total number of cells per pair of testes} = \text{(paired testes weight) (no. cells/gram)}$$

Calculation of Total Daily Sperm Cell Production. Daily sperm production (DSP) is the total number of sperm cells produced per day by two testes (Amann, 1970). The total number of sperm cells per two testes was determined from the above calculation. This represents the mature spermatozoa and spermatids from Stages VI, VII, and VIII of the seminiferous epithelial cycle. The time required for cells to advance through Stages VI, VII, and VIII is 3.4 days as shown by Amann and Lambiase (1969).

The 3.4 days was used as the time divisor of total sperm cell count to derive total daily sperm production.

Tissue Preparation for Histological Study

Testes. A testis was placed in Zenker-formol solution for fifteen minutes, removed and approximately 1 mm cut transversely from each pole of the organ with a sharp razor blade. The testis was replaced in the Zenker-formol solution for 24 hours, removed and washed under a continuous flow of cold tap water for 24 hours. The tissue was then placed in 70% ethanol and 4 micron sections cut and mounted on slides which were stained using the periodic acid-Schiff technique.

Experimental Design

The primary objective of this investigation was to find a method of administering low doses of testosterone which inhibit spermatogenesis without depressing libido or producing accessory sex organ hypertrophy in male rabbits for 3 months.

Experiment 1: Release of Testosterone-Tritium from Polydimethylsiloxane (PDS) Capsules into Distilled Water

The objectives of this experiment were: 1) to determine the variability of testosterone release from PDS capsules into distilled water, and 2) to establish the influence of capsule surface area on the rate of release. These objectives were accomplished by measuring the rate of release of testosterone-tritium from capsules with the following variations in external surface area; 225, 450, and 900 square millimeters with a thickness of 0.65 millimeter.

Six capsules, of each size, were filled with testosterone-tritium (Specific Activity = 1900 cpm/ug) and incubated individually in 100 ml of distilled water at room temperature. Samples were withdrawn for scintillation counting after 6, 12, and 24 hours, thus providing a total of 54 observations. The results were subjected to a 2 x 2 factorial analysis of variance (AOV) (Snedecor and Cochran, 1967). When the AOV revealed significant variation, the differences in treatment means were detected by Duncan's Multiple Range Test (Steel and Torrie, 1960).

Experiment 2: The Effect of Capsular Dimensions on the Concentration of Tritium in the Peripheral Plasma of Male Rabbits

The relationship of capsule surface area to the level of tritium in the peripheral plasma of intact male rabbits containing subcutaneously

placed testosterone-tritium filled capsules (SA = 1900 cpm/ug) was determined. The determination was made by measuring the appearance of tritium from testosterone-tritium filled PDS capsules with external surface areas of 225, 450, and 900 square millimeters and wall thickness of 0.65 millimeter. Each of the 3 capsule sizes were implanted subcutaneously into a total of 21 animals (7 replicates per dimension) and the concentration of tritium in a sample of peripheral plasma was determined at weekly intervals for one through six weeks for each animal. The 126 observations were subjected to a 2 x 2 factorial analysis of variance (AOV), (Snedecor and Cochran, 1967). When the AOV revealed significant variation, the differences in treatment means were detected by Duncan's Multiple Range Analysis (Steel and Torrie, 1960).

Experiment 3: The Effect of Subcutaneous or Intraperitoneal Placement of Testosterone-Tritium Filled PDS Capsules on the Concentration of Tritium in Peripheral Plasma of Male Rabbits

The appearance of tritium in the peripheral plasma of intact male rabbits containing testosterone-tritium filled capsules with external surface areas of 450 and 900 square millimeters and a wall thickness of 0.65 millimeter was determined. Each of the two capsule sizes were implanted either subcutaneously or intraperitoneally into a total of ten animals each (5 replicates per dimension). The concentration of tritium in a sample of peripheral plasma was determined at weekly intervals for one through six weeks for each animal. The sixty observations for each implantation site were subjected to a one-way analysis of variance to test for significant variance due to treatment.

Experiment 4: The Effect of Subcutaneous Testosterone Filled PDS Implants on Libido and Accessory Sex Organ Function of Castrate and Intact Male Rabbits; Sperm Production and Plasma Testosterone Concentration of Intact Male Rabbits

Forty-two male rabbits were trained to ejaculate into an artificial vagina. Twenty-one animals were anesthetized and bilateral castration performed. Twenty-one of the mature males were left intact. The castrate and intact groups were divided into three experimental treatment groups of seven animals each. These treatemnt groups received subcutaneous PDS implants as follows: 1) cholesterol filled 430 mm^2 capsule, 2) testosterone filled 215 mm^2 capsule and 3) testosterone filled 430 mm^2 capsule. The wall thickness of all implants was 0.245 mm.

Blood samples were taken at 30, 60, and 90 days post-implantation. At 70 days post-implantation sex behavior measurements were obtained; each animal was ejaculated using an artificial vagina, recording mounting time, ejaculation time, and seminal volume every other day for 20 days. At the end of the 90 day treatment period the animals were anesthetized, exsanguinated, euthanized and accessory sex organs collected from the intact animals. All organs were fixed for histology except one testis and epididymis from each animal which was weighed, homogenized in a Waring Blender, and counts made of spermatids and sperm cells.

The six treatment groups in this experiment provided data for sex behavior, and accessory sex organ weights and secretion of all the rabbits (6 x 7 = 42). The experimental design provided for a 2 x 2 factorial analysis of variance. The two factor analysis was a completely randomized design with implants designated the fixed variable and testes the random variable.

Testes weights, peripheral concentration of testosterone and daily sperm production were derived from the twenty-one intact rabbits (3 x 7 = 21). These results were subjected to one-way analysis of variance to test for significant differences due to the treatments.

When significant differences were indicated by either method of analysis of variance described above the treatment means were subjected to Duncan's Multiple Range Test, (Steel and Torrie, 1960).

Experiment 5: Effect of Surface Area of Testosterone-Filled PDS Capsules on Daily Sperm Production and Accessory Sex Organ Weight in Intact Male Rabbits

The purpose of this experiment was to elucidate the surface area of a PDS testosterone implant required to render all animals azoospermic without causing hypertrophy of accessory sex organs. Thirty-six intact male rabbits were divided into six treatment groups of six rabbits each. One group designated as controls received 400 mm^2 cholesterol filled PDS implants. The five treatment groups received 100, 200, 400, 800 and 1,600 mm^2 PDS testosterone filled implants respectively. All PDS capsules had a wall thickness of 0.245 mm. After 3 months the rabbits were sacrificed and body weight, paired testes weight, total daily sperm production, seminal vesicle, prostate, vesicular and bulbourethral gland weights recorded.

A 2 x 2 factorial analysis of variance was performed. The two factor analysis was a completely randomized design with implants designated the fixed variable.

CHAPTER IV

RESULTS

The primary objective of this investigation was to establish the size of testosterone filled Polydimethylsiloxane (PDS) capsule required to inhibit spermatogenesis in mature male rabbits without altering libido or causing accessory sex organ hypertrophy.

A series of five experiments was designed to accomplish this objective. The first experiment was to determine the influence of surface area of PDS capsules on the rate of testosterone-tritium release into distilled water. The results were used to predict the approximate dimensions of PDS testosterone filled capsules for subsequent *in vivo* experiments.

The second experiment was designed to determine the relationship of PDS capsule surface area to concentration of tritium in the peripheral plasma of intact male rabbits for one to six weeks following subcutaneous implantation of testosterone-tritium filled PDS capsules.

The third experiment was designed to compare peripheral plasma tritium concentration of intact male rabbits containing either subcutaneous PDS testosterone-tritium filled capsules or intraperitoneal PDS testosterone-tritium filled capsules for a forty-two day period.

The fourth experiment was designed to determine the approximate dimensions of testosterone filled PDS capsules placed subcutaneously in castrate male rabbits required to maintain peripheral plasma

testosterone concentration, male sex behavior, and accessory sex organ weight and secretion. The subcutaneous testosterone filled PDS capsules in intact male rabbits provided additional results on the effect of exogenous testosterone upon spermatogenesis.

The fifth experiment was designed to elucidate the surface area of testosterone filled PDS capsules required to render all animals azoospermic without causing hypertrophy of accessory sexual organs.

Experiment 1: Release of Testosterone-Tritium from Polydimethylsiloxane (PDS) Capsules into Distilled Water

Plate 1 shows the testosterone-tritium filled capsules (SA = 1900 cpm/ug testosterone) used to determine the release rate of the steroid into distilled water at room temperature. These capsules had a wall thickness of 0.65 mm and 225, 450, and 900 square millimeters of surface area respectively. The dark objects visible in each end of the capsules are bits of graphite that were used for identification of each capsule of each treatment group.

In vitro release of testosterone-tritium from these capsules into distilled water is shown in Table I. There is a linear increase in the amount of steroid released with respect to time for each capsule size. The release rate with respect to capsule surface area, although variable, indicates a direct relationship between rate of release and capsule surface area. Statistical analysis indicates significant variance ($P<0.01$) due to capsules surface area and time of incubation. This confirms experiments reported by Kincl et al., (1968).

Figure 1 shows the results of an experiment designed to prove that the passage of testosterone through the PDS membrane did not alter the

Plate 1. Polydimethylsiloxane capsules filled with testosterone-tritium, from left to right external surface area of 900, 450 and 225 mm^2. These capsules were used in experiments one, two and three. The dark objects in the ends of the capsules are pieces of graphite used for identification of each capsule.

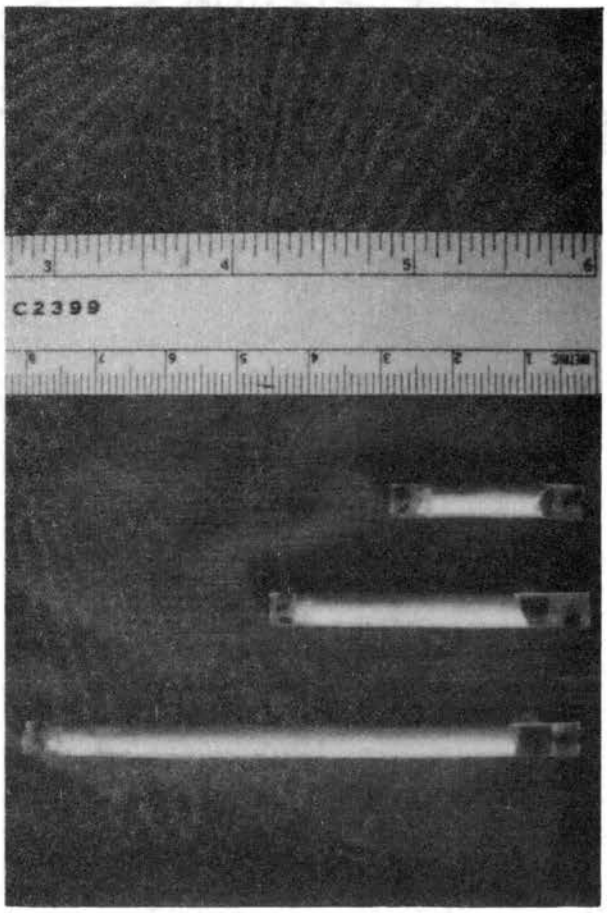

C2399

TABLE I

RELEASE OF TESTOSTERONE FROM POLYDIMETHYLSILOXANE CAPSULES INTO DISTILLED WATER AT ROOM TEMPERATURE

Capsule Surface Area (mm2)	Time (hours)		
	6	12	24
225	20 ± 1.6[a]	33 ± 2.3	62 ± 4.4
450	32 ± 4.1	53 ± 6.6	96 ± 7.8
900	96 ± 6.8	138 ± 9.7	212 ± 9.4

[a]Each value is expressed as ug testosterone/100 ml water and represents the mean ± standard error of six replicates.

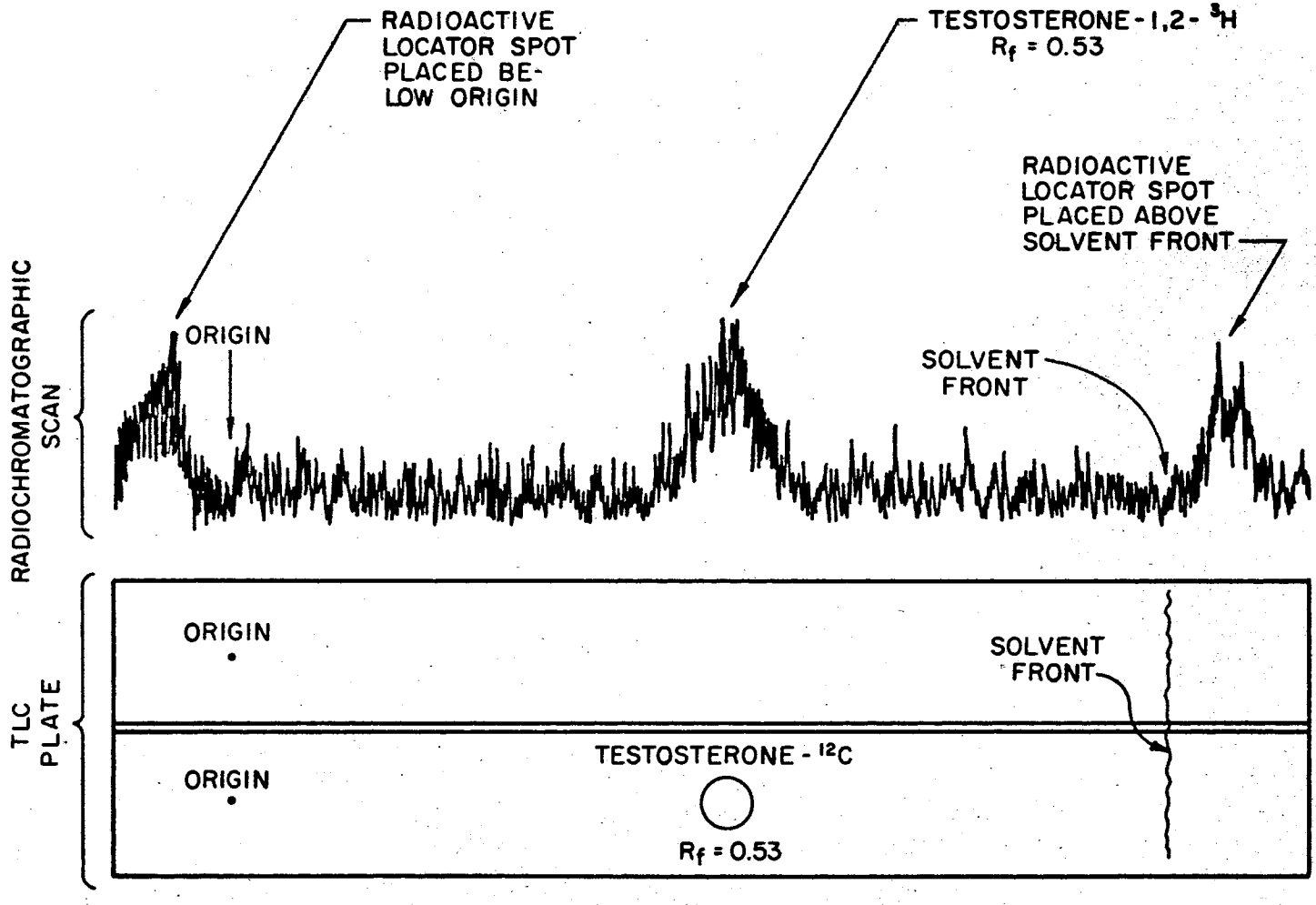


Figure 1. Radiochromatographic Scan of Concurrent Thin Layer Chromatography of Tritiated Material Extracted from Water Bathing PDS Testosterone Capsules and Authentic Testosterone-^{12}C. The solvent system was benzene:methanol (7:1 v:v).

structure of testosterone. Note that the tritium extracted from the water bathing the PDS capsules has the same chromatographic mobility on thin layer chromatography (benzene:methanol, 87.5:12.5 v/v) as authentic testosterone developed in adjacent lane.

These data suggest that testosterone-tritium was released in vitro from PDS capsules, that the release rate was continuous over a 24 hour period and dependent upon capsule surface area. Moreover, passage through PDS does not alter the steroid's chromatographic mobility upon thin layer chromatography.

Experiment 2: The Effect of Capsular Dimensions on the Concentration of Tritium in the Peripheral Plasma of Male Rabbits

The testosterone-tritium filled capsules used in Experiment 1 were implanted subcutaneously into intact male rabbits.

The results of this experiment seen in Table II, show a direct relationship between capsule surface area and the amount of tritium detectable in the peripheral plasma over a 6 week period. Increasing surface area from 225 mm^2 to 900 mm^2 resulted in increased tritium concentration in the peripheral plasma. There was a significant difference ($P<0.05$) in the plasma tritium concentrations of animals receiving the 225 mm^2 compared to the 900 mm^2 capsules. There was no significant difference between 225 and 450 or 450 and 900 mm^2. Moreover, there was no significant difference due to time.

These results suggest that testosterone-tritium was released in vivo from PDS capsules and that increasing the capsule surface area resulted in increasing plasma tritium concentrations. Moreover, this relationship existed for 7 weeks.

TABLE II

TRITIUM CONCENTRATION IN THE PERIPHERAL PLASMA OF INTACT MALE RABBITS CONTAINING SUBCUTANEOUSLY PLACED PDS TESTOSTERONE-TRITIUM FILLED CAPSULES

Capsule Surface Area (mm^2)	Time (weeks)						
	1	2	3	4	5	6	7
225	269 ± 12[a]	256 ± 56	196 ± 20	152 ± 27	162 ± 20	181 ± 31	182 ± 24
450	331 ± 29	350 ± 22	307 ± 26	250 ± 30	252 ± 31	347 ± 26	277 ± 27
900	797 ± 198	741 ± 89	690 ± 74	746 ± 81	723 ± 117	687 ± 93	661 ± 158

[a]The data is expressed as dpm tritium/ml plasma. Each value represents the mean ± or standard error of seven rabbits.

Experiment 3: The Effect of Subcutaneous or Intraperitoneal Placement of Testosterone-Tritium Filled PDS Capsules on the Concentration of Tritium in Peripheral Plasma of Male Rabbits

Testosterone-tritium filled PDS capsules were placed subcutaneously and intraperitoneally into mature male rabbits. Blood samples were collected weekly for 6 weeks and the peripheral plasma tritium concentration measured. The capsules used in this experiment were the same ones used in Experiment 2.

The results in Table III show that there was no significant difference due to time and no significant difference in tritium concentration in peripheral plasma of rabbits containing subcutaneous or intraperitoneal capsules.

These results suggest the subcutaneous placement of testosterone-tritium filled capsules provides testosterone-tritium release similar to intraperitoneal placement of capsules. Since the same capsules were used in experiments 2 and 3 it also shows that a given PDS capsule can release testosterone-tritium at a relatively constant rate over a 13 week period *in vivo*.

Taken together, the results have shown that testosterone-tritium was released from PDS capsules *in vitro* and *in vivo*. The *in vivo* testosterone-tritium release was relatively constant over a 13 week period assuming plasma tritium concentration is directly related to testosterone release rate.

TABLE III

TRITIUM CONCENTRATION IN THE PERIPHERAL PLASMA OF INTACT MALE RABBITS CONTAINING SUBCUTANEOUS OR INTRAPERITONEAL PDS TESTOSTERONE TRITIUM FILLED IMPLANTS

Capsule Surface Area (mm^2)	Site	Time (weeks)					
		1	2	3	4	5	6
450	S.C.	293 ± 25[a]	304 ± 22	293 ± 30	305 ± 15	277 ± 37	256 ± 28
	I.P.	343 ± 65	375 ± 24	326 ± 30	366 ± 46	264 ± 28	348 ± 30
900	S.C.	727 ± 96	327 ± 13	708 ± 60	578 ± 24	600 ± 13	658 ± 64
	I.P.	786 ± 94	709 ± 64	581 ± 92	523 ± 115	726 ± 117	846 ± 108

[a]The data is expressed as dpm of tritium/ml of plasma. Each value represents the mean ± standard error of seven rabbits.

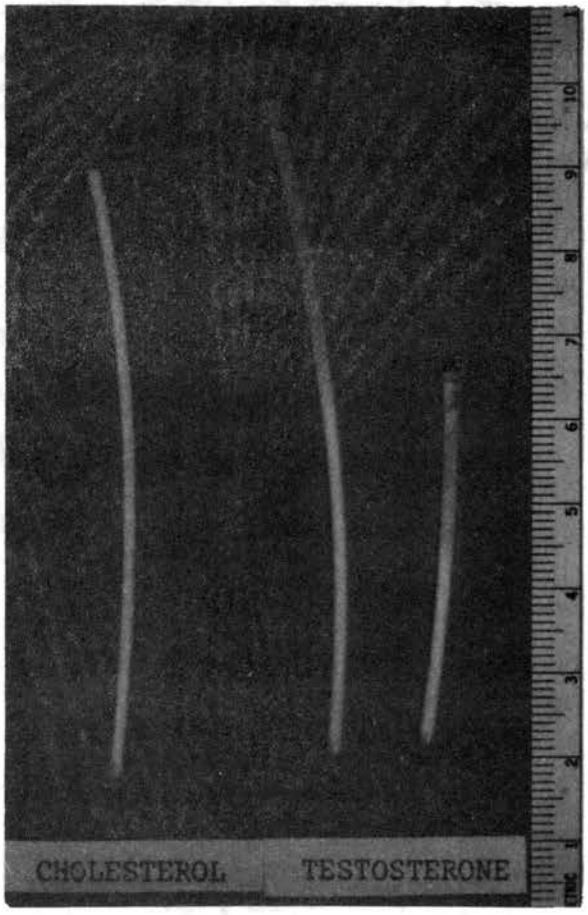
Experiment 4: The Effect of Subcutaneous Testosterone Filled PDS Implants on Libido and Accessory Sex Organ Function of Castrate and Intact Male Rabbits; Sperm Production and Plasma Testosterone Concentration of Intact Male Rabbits

Experiments 1 and 3 failed to answer two questions: 1) what size testosterone filled subcutaneous PDS implant is required to maintain libido and accessory sex organ weight and secretion at control levels in castrate male rabbits and 2) what size testsoterone filled subcutaneous PDS implant is required to produce azoospermia in all treated males without altering sexual behavior or causing accessory sex organ hypertrophy?

Plate II shows typical steroid-containing PDS capsules used in Experiment 4. These capsules had a wall thickness of 0.245 mm and 215 and 430 square millimeters of surface area respectively. The capsules were implanted subcutaneously in rabbits for ninety days prior to the time this photograph was made. Note the uniform density of the cholesterol capsules while the capsules containing testosterone appear to be only partially filled. This suggests little or none of the cholesterol diffused out of the PDS capsules into the control rabbits in which they were implanted, and provides visual evidence of the ability of testosterone to diffuse out of the PDS capsules.

Androgenic Effect of 215 and 430 mm^2 Subcutaneous Testosterone Implants in Castrate Male Rabbits. This portion of experiment 4 evaluated the capacity of testosterone, chronically administered via subcutaneous PDS capsules, to maintain libido and accessory sex organ size and function in castrate male rabbits over a period of 90 days.

Plate 2. Steroid containing polydimethylsiloxane capsules with external surface areas 430, 430 and 215 mm^2. These capsules were used in experiment 4. Note the different density in the upper portion of the testosterone containing capsules. This picture was made following 90 days subcutaneous placement in rabbits.


CHOLESTEROL
TESTOSTERONE

Sexual Behavior. The effect of testosterone filled PDS implants on the sex behavior of castrate male rabbits is shown in Table IV. Castration for 70 days abolished sex behavior in male rabbits (0 score) as expected. Implantation of 215 mm^2 testosterone PDS implants significantly increased the sex behavior score to 6.0 while those receiving 430 mm^2 testosterone PDS implants showed a further increase of sex behavior score to 9.8. There was no significant difference between the scores of intact rabbits (8.4) and castrate rabbits which received the 430 mm^2 testosterone PDS implant.

These results suggest that the sexual behavior of castrate male rabbits was maintained at normal levels by exogenous testosterone supplied by testosterone filled PDS implants with a surface area of 430 mm^2 with wall thickness of 0.245 mm.

Accessory Sex Organ (ASO) Weight. The results in Table V show the effect of subcutaneous testosterone filled PDS implants on the weight of seminal vesicles, prostate, vesicular and bulbouretheral glands of castrate male rabbits.

The removal of testicular androgen, via castration, resulted in a marked decrease in weight of all the accessory sex organs mentioned above.

Accessory sex organ weights of castrate rabbits receiving testosterone PDS implants showed response proportionate to the surface area of testosterone PDS capsules implanted. Rabbits receiving the 215 mm^2 implants had ASO weights 58, 60, 67 and 68 percent of that observed in intact rabbits. Accessory sex organ weights of rabbits receiving 430 mm^2 testosterone PDS implants were not significantly different from the intact rabbits.

TABLE IV

EFFECT OF PDS TESTOSTERONE IMPLANTS ON THE SEX BEHAVIOR OF CASTRATE MALE RABBITS

Contents and Size of Implants	Testes	Sex Behavior Score (0-10)
Cholesterol 430 mm^2	-	0.0[a]
Cholesterol 215 mm^2	-	6.0 ± 1.7
Testosterone 430 mm^2	-	9.8 ± 0.13
Testosterone 430 mm^2	+	8.4 ± 0.85

[a]Each value represents the mean ± the standard error of seven rabbits following subcutaneous placement for ninety days.

TABLE V

EFFECT OF PDS TESTOSTERONE IMPLANTS ON THE WEIGHTS OF ACCESSORY SEX ORGANS OF CASTRATE MALE RABBITS

Contents and Size of Implants	Testes	Organ Weights			
		Seminal Vesicles	Prostate Gland	Vesicular Gland	Bulbourethral Gland
Cholesterol 430 mm^2	-	135 ± 10[a]	113 ± 20	129 ± 29	216 ± 50
Cholesterol 430 mm^2	+	508 ± 70	693 ± 60	807 ± 70	622 ± 90
Testosterone 215 mm^2	-	297 ± 40	415 ± 50	539 ± 60	424 ± 30
Testosterone 430 mm^2	-	476 ± 90	572 ± 40	778 ± 90	616 ± 60

[a]Each value represents the mean ± the standard error of seven rabbits following subcutaneous placement for ninety days.

These results confirm the fact that accessory sex organ weight is dependent upon testosterone (Mann et al., 1949). It also suggests that the 430 mm^2 testosterone implant releases adequate amounts of testosterone to maintain ASO weight similar to control rabbits.

Accessory Sex Organ Secretion. That accessory sex organ secretion is maintained by subcutaneous PDS testosterone implants is shown in Table VI. Castration for 90 days reduces seminal plasma volume, citric acid and fructose concentration to zero. The castrate male rabbits which received 215 mm^2 PDS testosterone implants produced seminal plasma with significantly ($P<0.05$) lower citric acid and fructose concentrations than the castrate males which contained 430 mm^2 PDS testosterone implants or the intacct males which contained cholesterol filled implants. There was no significant difference in the seminal plasma volume, citric acid or fructose concentrations of the castrate males receiving 430 mm^2 PDS testosterone implants and the intact males receiving cholesterol PDS implants.

These results confirm that the 430 mm^2 testosterone PDS implant releases adequate amounts of testsoterone to maintain ASO weight and secretion.

Antispermatogenic Effect of Testosterone PDS Implants in Intact Male Rabbits. The second part of experiment 4 involved an evaluation of the ability of testosterone, administered via subcutaneous PDS capsules, to inhibit spermatogenesis in intact male rabbits without changing libido or producing accessory sex organ hypertrophy.

This evaluation was based on changes in testicular morphology and spermatozoa production, sex behavior, accessory sex organ size and

TABLE VI

EFFECT OF PDS TESTOSTERONE IMPLANTS ON SECRETORY FUNCTION OF ACCESSORY SEX ORGANS OF CASTRATE MALE RABBITS

Contents and Size of Implants	Testes	Seminal Plasma		
		Volume (ml)	Citric Acid Concentration (mg/ml)	Fructose Concentration (mg/ml)
Cholesterol 430 mm^2	-	0.0	0.0	0.0
Cholesterol 430 mm^2	+	0.43 ± 0.08	1.73 ± 0.30	2.30 ± 0.34
Testosterone 215 mm^2	-	0.39 ± 0.15[a]	0.62 ± 0.23	0.93 ± 0.14
Testosterone 430 mm^2	-	0.59 ± 0.05	2.11 ± 0.23	2.46 ± 0.52

[a]Each value represents the mean ± the SE of seven rabbits following subcutaneous placement of the implants for ninety days.

function and peripheral plasma testosterone concentration.

Testicular Function. Spermatogenic function was evaluated by testing the effect of PDS testosterone capsules on testicular histology, size and spermatozoan production.

Testicular Histology. Plate 3 is a photomicrograph of a representative field of testis sections from rabbits receiving cholesterol PDS capsules. Note the presence of mature spermatids ready to detach from germinal eptihelium becoming free testicular spermatozoa in the tubule lumen.

Plate 4 shows a representative field of testis sections from rabbits receiving the 430 mm^2 PDS testosterone implants. Note the reduced diameter and marked histological changes in the seminiferous tubules. Most of the inhibited tubules are devoid of all spermatids, with a reduced number of pachtene spermatocytes and secondary spermatocytes.

Perusal of the histological sections leads one to believe that the 430 mm^2 subcutaneous testosterone implants seriously impair spermatogenic function of rabbit testes.

Testis Size. The results in Table VII show that paired testis weights of rabbits receiving 215 mm^2 PDS testosterone implants for ninety days were significantly ($P<0.01$) reduced from paired testis weights of those receiving cholesterol implants. When the capsule size was increased to 430 mm^2 the testes weight was reduced even further (6.14 g to 1.56 g).

Spermatozoa Production. Rabbits receiving the 215 mm^2 PDS testosterone implants showed a marked reduction ($P<0.01$) in daily sperm

Plate 3. A photomicrograph of a cross-section of seminiferous tubules from a mature rabbit that received a 430 mm^2 PDS cholesterol implant for 90 days. Note the size of the tubule in relation to field of view, and the presence of mature spermatids. Magnification 250X.

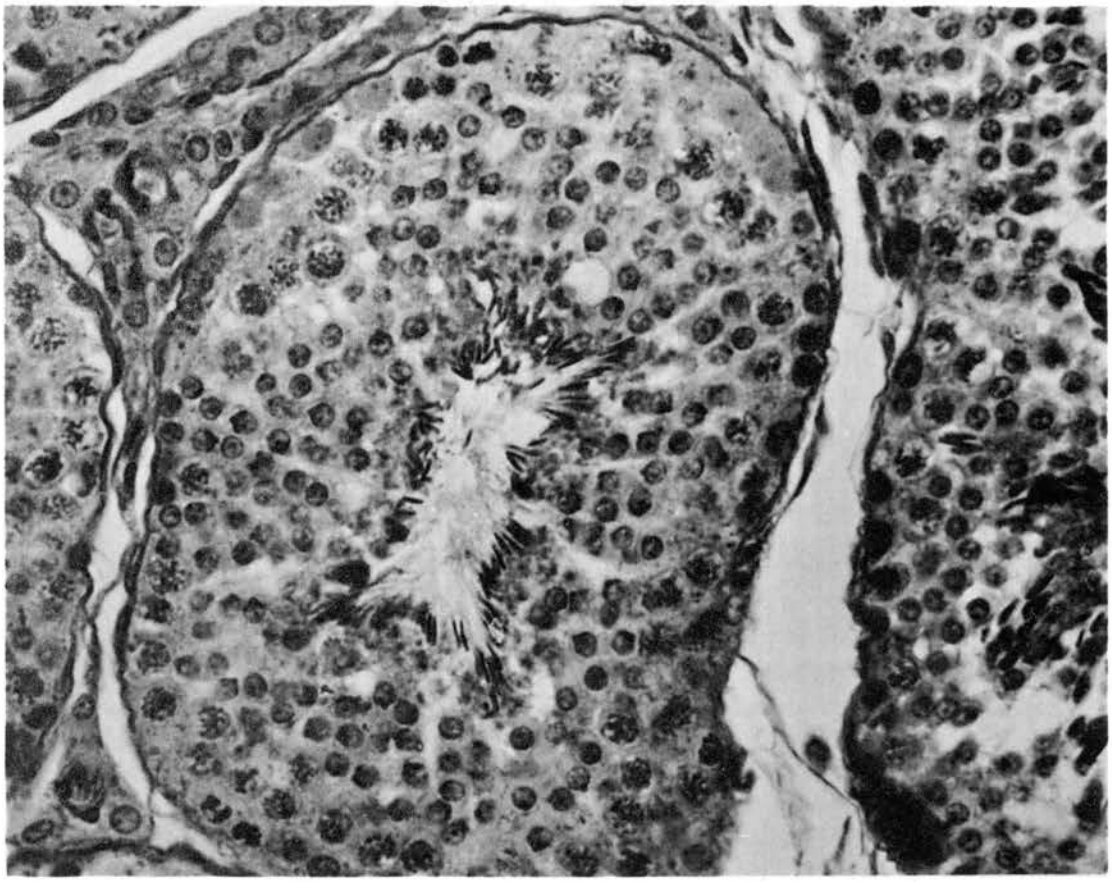


Plate 4. A photomicrograph of a seminiferous tubule cross-section from a mature rabbit that received a 430 mm^2 PDS testosterone implant for 90 days. Note the size of the tubules in relation to the field of view, the absence of spermatids. Magnification 250X.

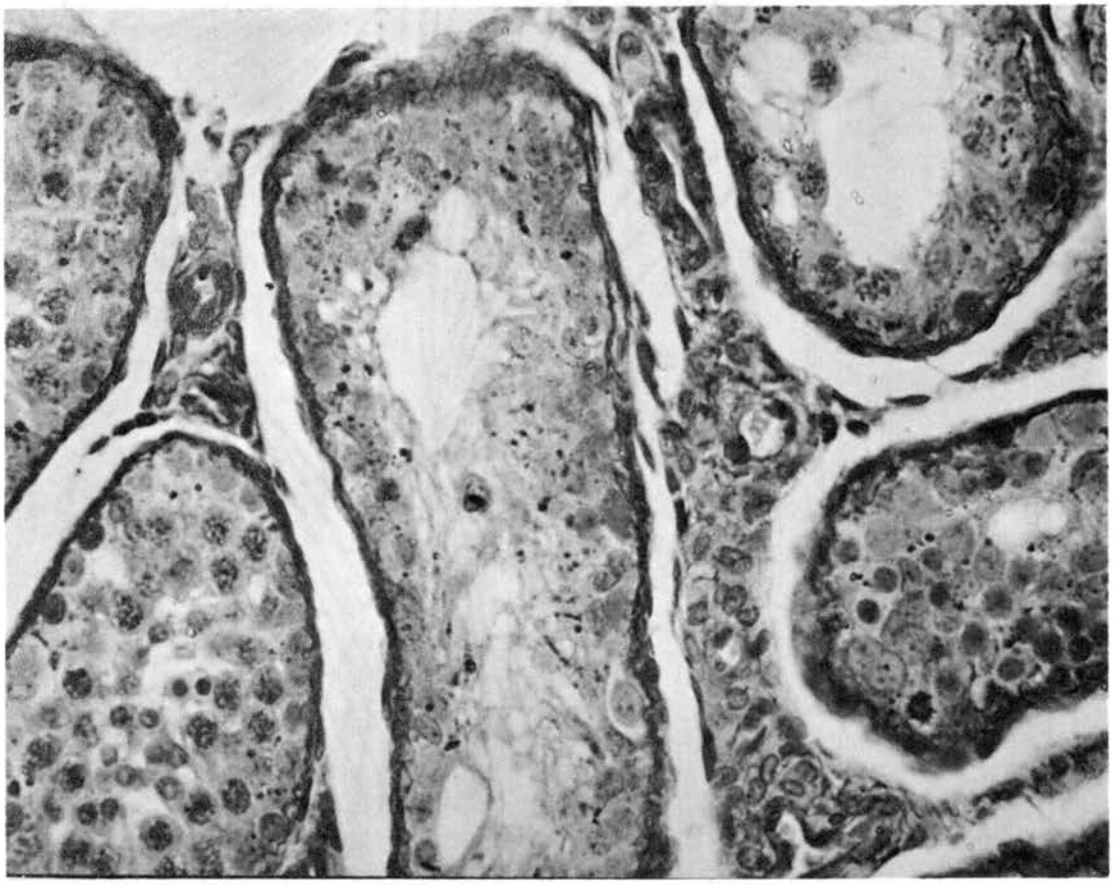


TABLE VII

EFFECT OF PDS TESTOSTERONE IMPLANTS ON TESTES FUNCTION

Contents and Size of Implants	Size of Testes and Spermatozoa Production	
	Paired Testes Weight (g)	Total Daily Sperm Production ($x10^6$)
Cholesterol 430 mm^2	6.14 ± 0.61[a]	214.9 ± 24.4
Testosterone 215 mm^2	3.62 ± 0.38	123.5 ± 29.1
Testosterone 430 mm^2	1.56 ± 0.36	16.0 ± 14.9

[a]Each value represents the mean ± the SE of seven rabbits following subcutaneous placement of the implants for ninety days.

production when compared to control rabbits. Five of seven rabbits which received the 430 mm^2 PDS testosterone implant had sperm counts of zero. The 16 million total daily sperm production shown in Table VII resulted from impaired spermatozoa production by two rabbits in the treatment group.

These results show that the 430 mm^2 testosterone filled PDS capsules implanted subcutaneously for 90 days in mature male rabbits will completely inhibit spermatozoa production in five of seven rabbits.

Sexual Behavior

That exogenous testosterone administered continuously via PDS testosterone implants in amounts sufficient to inhibit spermatogenesis in five of seven rabbits will not significantly change sexual behavior is shown in Table VIII. Although the intact male rabbits receiving 430 mm^2 PDS testosterone capsules consistently scored the highest sexual behavior, there was no significant difference in scores of intact males receiving cholesterol filled, 215 mm^2 or 430 mm^2, testosterone filled PDS capsules.

Accessory Sex Organ Weight

The data in Table IX show the effect of exogenous testosterone, administered via PDS capsules for 90 days, on the weight of seminal vesicles, prostate, vesicular and bulbouretheral glands in intact rabbits.

The heaviest accessory sex organs were those of the cholesterol implanted control rabbits. None of the animals receiving testosterone PDS implants had accessory sex organs which exhibited hypertrophy.

TABLE VIII

EFFECT OF PDS TESTOSTERONE IMPLANTS IN SEXUAL BEHAVIOR OF INTACT MALE RABBITS

Contents and Size of Implants	Sex Behavior Score (0-10)
Cholesterol 430 mm^2	8.65 ± 0.85a
Testosterone 215 mm^2	8.07 ± 0.97
Testosterone 430 mm^2	9.90 ± 0.03

[a]Each value represents the mean ± the SE of seven rabbits following subcutaneous placement of the implants for ninety days.

TABLE IX

EFFECT OF PDS TESTOSTERONE IMPLANTS ON WEIGHTS OF ACCESSORY SEX ORGANS OF INTACT MALE RABBITS

Contents and Size of Implants	Organ Weights (mg)			
	Seminal Vesicles	Vesicular Gland	Prostate Gland	Bulbourethral Gland
Cholesterol 430 mm^2	508 ± 70[a]	807 ± 70	693 ± 60	622 ± 90
Testosterone 215 mm^2	358 ± 30	762 ± 80	458 ± 30	527 ± 20
Testosterone 430 mm^2	398 ± 30	804 ± 80	510 ± 40	608 ± 70

[a]Each value represents the mean ± the SE of seven rabbits following subcutaneous placement of the implants for ninety days.

Moreover, there were no significant differences in ASO weights between any treatment groups.

These results suggest the 215 and 430 mm^2 testosterone filled PDS capsules will not cause accessory sex organ hypertrophy when placed in intact male rabbits for 90 days.

Accessory Sex Organ Secretion

The results in Table X show that subcutaneous implantation of testosterone PDS capsules for 90 days in intact male rabbits did not change the secretory function of the accessory sex organs as measured by seminal volume, citric acid concentration and fructose concentration of the seminal plasma. This suggests that the sizes of PDS testosterone capsules used in this experiment will not alter the function of accessory sex organs when placed subcutaneously for 90 days in intact male rabbits.

Peripheral Plasma Testosterone Concentration

A basic thesis of this investigation was that it is possible to administer exogenous testosterone in dosages that inhibit hypophyseal gonadotrophic hormone release, resulting in decreased testicular testosterone secretion and sperm production. Theoretically the exogenous testosterone could maintain peripheral testosterone at concentrations sufficient to maintain libido without causing accessory sex organ hypertrophy.

The results in Table XI show that peripheral plasma testosterone concentration of intact male rabbits containing PDS testosterone implants is not significantly different that the concentration of testosterone in the peripheral plasma of rabbits implanted with PDS cholesterol capsules.

TABLE X

EFFECT OF PDS TESTOSTERONE IMPLANTS ON SECRETORY FUNCTION OF ACCESSORY SEX ORGANS OF INTACT MALE RABBITS

Contents and Size of Implant	Seminal Plasma		
	Volume (ml)	Citric Acid Concentration (mg/ml)	Fructose Concentration (mg/ml)
Cholesterol 430 mm^2	0.60 ± 0.08	1.73 ± 0.30	2.30 ± 0.34
Testosterone 215 mm^2	0.52 ± 0.04	1.64 ± 0.24	2.37 ± 0.30
Testosterone 430 mm^2	0.63 ± 0.04	1.62 ± 0.17	1.74 ± 0.09

[a]Each value expressed is the mean ± the standard error of the mean of seven replicates.

TABLE XI

EFFECT OF PDS TESTOSTERONE IMPLANTS ON PERIPHERAL PLASMA TESTOSTERONE CONCENTRATION IN INTACT MALE RABBITS

Contents and Size of Implants	Peripheral Plasma Testosterone ng/ml
Cholesterol 430 mm^2	2.14 ± 0.75[a]
Testosterone 215 mm^2	1.45 ± 0.30
Testosterone 430 mm^2	1.66 ± 0.13

[a]Each value represents the mean ± the SE of seven rabbits following subcutaneous placement of the implants for ninety days.

These results show that testosterone was maintained at a constant level in intact rabbits from two sources (PDS testosterone implants and testes) and suggests that the testicular contribution was decreased in animals receiving the 215 and 430 mm^2 PDS testosterone implants.

The fact that only five of seven rabbits receiving the 430 mm^2 PDS testosterone implants were azoospermic suggests that larger implants are required to completely inhibit spermatogenesis in rabbits.

Experiment 5: Effect of Surface Area of Testosterone Filled PDS Capsules on Daily Sperm Production and Accessory Sex Organ Weight in Intact Male Rabbits

The objective of this experiment was to elucidate the surface area of PDS testosterone implant necessary to render all animals azoospermic without causing hypertrophy of accessory sex organs.

Effect of PDS Testosterone Implants on Testes Function. The data in Table XII show the effect of testosterone filled PDS implants on testicular function.

Testis Size. The results in Table XII show that paired testis weights were significantly decreased ($P<0.01$) in rabbits receiving testosterone PDS implants with 400, 800, or 1600 mm^2 surface area, but that there was no significant decrease in rabbits receiving testosterone PDS implants with surface area of 100 or 200 mm^2.

Spermatozoa Production. That total daily sperm production was decreased significantly ($P<0.01$) with increased surface area of testosterone PDS implants is also shown in Table XII. Animals receiving the 800 mm^2 testosterone PDS implants were all azoospermic.

TABLE XII

EFFECT OF PDS TESTOSTERONE IMPLANTS ON TESTES FUNCTION OF RABBITS

Content and Size of Implants	Size of Testes and Spermatozoa Production	
	Paired Testes Weight (g)	Total Daily Sperm Production ($x10^6$)
Cholesterol 400 mm^2	4.89 ± 0.58	210.0 ± 38.4
Testosterone 100 mm^2	4.85 ± 0.20	193.0 19.3 ± 17.4
Testosterone 200 mm^2	4.53 ± 0.62	154.0 ± 36.2
Testosterone 400 mm^2	1.91 ± 0.62	34.0 ± 19.0
Testosterone 800 mm^2	0.65 ± 0.11	0.0 ± 0.0
Testosterone 1600 mm^2	1.00 ± 1.07	7.0 ± 4.2

[a]Each value represents the mean ± the standard error of six rabbits following subcutaneous placement for ninety days.

Effect of PDS Testosterone Implants on the Weights of Accessory Sex Organs of Intact Rabbits. The results in Table XIII show that seminal vesicle, prostate, vesicular and bulbouretheral gland weights were not significantly altered due to surface area of PDS testosterone implants until the surface area was increased to 1600 mm^2.

Taken together the results of experiment 5 suggest that spermatogenesis can be inhibited in male rabbits without causing accessory sex organ hypertrophy.

TABLE XIII

EFFECT OF PDS TESTOSTERONE IMPLANTS ON THE WEIGHTS OF ACCESSORY SEX ORGANS OF INTACT RABBITS

Contents and Size of Implants	Organ Weights (mg)			
	Seminal Vesicles	Prostate Gland	Vesicular Gland	Bulbourethral Gland
Cholesterol 400 mm^2	312 ± 47[a]	611 ± 68	642 ± 60	663 ± 173
Testosterone 100 mm^2	306 ± 33	741 ± 163	788 ± 77	620 ± 51
Testosterone 200 mm^2	388 ± 47	638 ± 57	652 ± 93	569 ± 72
Testosterone 800 mm^2	311 ± 47	479 ± 41	565 ± 68	484 ± 54
Testosterone 1600 mm^2	1015 ± 16	661 ± 36	955 ± 112	888 ± 84

[a]Each value represents the mean ± the standard error of six rabbits following subcutaneous placement for ninety days.

CHAPTER V

DISCUSSION

Numerous investigations have shown that steroidal hormones, including testosterone pass through polydimethylsiloxane membranes at predictable rates (Dziuk and Cook, 1966; Kincl et al., 1968; Moon and Bunge, 1968; Chang and Kincl, 1970; Hwang et al., 1970). However, these investigators did not evaluate the rate of testosterone release critically enough to allow us to ascertain the rates of testosterone release required for our experiments.

Kincl et al., (1968) reported that testosterone was released from PDS capsules with wall thickness of 0.1 mm at the rate of 317 ug/24 hrs/100 mm^2 into distilled water. Extrapolation of results obtained with the 900 mm^2, 0.65 mm thick, PDS testosterone capsules shown in Table I of this report, indicates a testosterone release rate of 156 ug/24 hrs/100 mm^2 for a 0.1 mm PDS membrane. These release rates obtained by the two laboratories are of the same order of magnitude and the slight difference can probably be explained on the basis of different lots of Silastic tubing (Hwang et al., 1970) or different methods of pakcing the Silastic tubing with crystalline testosterone. Our experience indicates that the elastic properties of Silastic tubing allow distension of capsule walls during filling with a crystalline material and that sharp steroid crystals lacerate tubing walls if the crystals are packed into the tubing with excessive force.

The fact that our results prove that there is a direct relationship between capsule surface area and rate of testosterone movement out of PDS capsules *in vitro*, suggests that different dosages of testosterone could be administered to an animal by varying the surface area of the PDS membrane.

The fact that the tritium extracted from the distilled water bathing the testosterone-tritium filled PDS capsules has the same chromatographic mobility as authentic testosterone (Figure 1) is proof that testosterone passes through PDS membranes unchanged. This is important if we are to use testosterone filled PDS capsules to inhibit spermatogenesis in intact rabbits. However, these experiments do not prove that testosterone will be released in sufficient quantities to inhibit spermatogenesis in rabbits. Moreover, they do not prove that there is a relationship of capsule surface area to testosterone release from subcutaneous testosterone PDS implants.

Moon and Bunge (1968) reported that Silastic capsules containing testosterone release sufficient quantities of testosterone to maintain the growth rate of accessory sex organs of castrate rats for sixteen weeks. These results although encouraging could not be used to support our experiments since Moon and Bunge used rats, since no quantitative rate of steroid release was determined and since all the rats receiving Silastic testosterone capsules were castrated.

It was postulated that monitoring the tritium levels in peripheral plasma of rabbits containing PDS testosterone implants would be a simple and effective means to determine the relative rate of testosterone release from PDS capsules of various dimensions *in vivo*.

The author realizes that testosterone in the circulating blood of

animals is rapidly metabolized (Leach et al., 1956; Rhynes, 1971). The results in Table XIV (Appendix A) show that testosterone-tritium represents only 1 to 2 percent of the total plasma tritium present in rabbits receiving subcutaneous testosterone-tritium PDS implants. However, since we are interested only in the release of tritium from the PDS capsules the important question is whether or not there is a relationship between peripheral plasma tritium concentration and capsule dimension after a period of several weeks.

Results of experiment 2 (Table II) show that increasing PDS testosterone implant size from 225 mm^2 to 900 mm^2 in intact male rabbits produced a significant ($P>0.05$) increase in peripheral plasma tritium concentration. Moreover, the relationship existed throughout the 7 week experimental period without a significant decrease in plasma tritium concentration due to time. Taken together these results suggested that steroid release was related to surface area of the capsule and was continuous over the 7 week period.

Experiment 3 was designed to answer the question of whether the site of capsule implantation influences rate of testosterone-tritium passage through the PDS capsule wall. The subcutaneous site was chosen because it provides a limited absorbing surface of tissue while the peritoneal cavity offers a large absorbing surface from which drugs enter the circulation rapidly (Fingl and Woodbury, 1970). The comparison of release rate versus site of implantation was not intended to provide evidence to select the most efficacious site for PDS testosterone implants, but to demonstrate that the factor limiting testosterone-tritium release from subcutaneous PDS implants was the surface area of the capsule. The intraperitoneal site of implantation was not

used in experiments where we attempted to inhibit spermatogenesis since absorption of drugs from the peritoneal cavity takes place primarily through the visceral rather than the pareital peritoneum or lymphatics (Leach et al., 1956; Fingl and Woodbury, 1970) resulting in rapid drug metabolism.

No significant difference in plasma tritium concentration was found in rabbits containing subcutaneous or intraperitoneal testosterone-tritium PDS capsules (Table III). The results of experiment 3 show (Table III) that there is no significant difference in plasma tritium concentration from a given capsule size, with time. Since the same PDS testosterone capsules were used in experiments 2 and 3, these results suggest that a continuous release of testosterone-tritium was achieved for thirteen weeks.

Taken together the results of experiments 1, 2, and 3 have shown that testosterone is released from PDS capsules in vitro and in vivo, that release is continuous for up to 13 weeks and that the rate of release in these experiments was influenced primarily by the external surface area of the PDS capsules. This information along with information from experiments reported by Kincl et al., (1968) was used to calculate the capsule dimensions required for subsequent in vivo experiments. Several questions remain unanswered.

What size of PDS testosterone implant is required to maintain male sexual behavior at control levels in castrate male rabbits? Castration for 70 to 90 days abolished sexual behavior in male rabbits (Table IV) measured by each rabbit's proficiency to mount, thrust, and/or ejaculate into an artificial vagina within a specified time. Rabbits receiving 430 mm^2 (0.245 mm wall thickness) PDS testosterone implants had

sexual behavior scores that were not significantly different from the scores of intact controls receiving cholesterol implants (Table IV). These results indicated that enough testosterone was released from the 430 mm^2 capsules to maintain libido of castrate male rabbits at control levels. The fact that the 215 mm^2 capsules only partially restored libido of castrate rabbits supports the contention that the amount of testosterone released from PDS capsules is dependent upon surface area. These results compare favorably with results reported by Macmillan and co-workers (1969), who reported restoration of normal libido in castrate male rabbits with 0.5 mg of testosterone propionate administered subcutaneously in corn oil every second day.

What size of PDS testosterone implant is required to maintain accessory sex organ size and secretion at control levels in castrate rabbits? Castration resulted in a marked decrease in weight of seminal vesicles, prostate, vesicular and bulbourethral glands of rabbits (Table V). Rabbits receiving 430 mm^2 PDS testosterone implants had accessory sex organ weights that were not significantly different from the accessory sex organ weights of intact controls receiving cholesterol implants (Table V). These results indicated that enough testosterone was released from the 430 mm^2 capsules to maintain accessory sex organ weights of castrate male rabbits at control levels, moreover, the weights of accessory glands of the rabbits containing 430 mm^2 testosterone implants did not exceed the accessory gland weights of the intact control rabbits. Further evidence of the dependence of steroid release rate on capsule surface area is provided since rabbits receiving the 215 mm^2 testosterone capsules had accessory sex organ weights significantly lower than those of the intact control rabbits and rabbits

receiving 430 mm^2 testosterone capsules. These results are in agreement with those of Price (1947) confirming that accessory sex organ weights are regulated by the amount of testosterone available.

The effect of testosterone on the maintenance of size and function of seminal vesicles, prostate, vesicular and bulbourethral glands is well established (Mann, 1956; Price and Williams-Ashman, 1961; Macmillan et al., 1969). That the secretion of both citric acid and fructose by the male accessory sex organs occurs only in the presence of the male sex hormone and is subject to fluctuations in response to decreased or increased activity of testosterone in the male is documented (Mann et al., 1949). Testosterone supplied to castrate male rabbits via 430 mm^2 PDS testosterone implants maintained the citric acid and fructose concentrations of seminal plasma to the levels of intact control rabbits while the amount of testosterone supplied to rabbits via 215 mm^2 capsules did not (Table VI). Thus taken together the results of experiment 4 have shown that testosterone from testes of male rabbits can be replaced via 430 mm^2 testosterone filled PDS capsules placed subcutaneously. These observations are interpreted to mean that the amount of testosterone released from the 430 mm^2 capsule is approximately the same as that amount reaching the systemic circulation in intact male rabbits.

There may be a different level of testosterone required by individual target organs including the hypothalamus, accessory sex organs and germinal epithelium. Albert (1961) and Woods and Simpson (1961) suggest that less testosterone is required for maintenance of accessory sex organs than is required for germinal epithelium to complete spermatogenesis. Davidson (1967) concluded that the hypothalamus contains

receptors sensitive to high or low circulating levels of testosterone, with high levels of testosterone responsible for diminished release of gonadotrophic hormones from the pituitary.

The results of this study suggest that a 430 mm^2 PDS testosterone implant may release adequate amounts of testosterone to maintain brain and accessory sex organ function in male rabbits, inhibit release of anterior pituitary gonadotrophins and reduce high local levels of endogenous testosterone in the testes resulting in aspermatogenesis. Therefore, the next series of questions to be answered dealt with the antispermatogenic effect of PDS testosterone implants in intact male rabbits.

What effect does PDS testosterone implants have on the morphology of germinal epithelium, size of the testes and total daily sperm production of rabbits? Rabbits containing 430 mm^2 testosterone capsules exhibited greater reduction in germinal cells (Plate IV) and testicular weight (Table VII) than rabbits containing 215 mm^2 testosterone capsules. The germinal epithelial changes were very similar to those observed in hypophysectomized rats by Ludwig (1950) and rabbits reported by Albert (1961). Rabbits containing 430 mm^2 testosterone capsules showed the largest drop in sperm production. Five of seven rabbits receiving 430 mm^2 capsules had no detectable spermatozoa in tissue homogenates of testicular parenchyma after the capsules had been in place for 90 days. The 16 million daily sperm production shown in Table VII resulted from two rabbits with impaired spermatozoa production. There are no published reports of research to compare with these findings.

We were aware of the fact that if this method were to be an effective male contraceptive it must cause azoospermia without altering

sexual behavior and accessory sex organ morphology and secretion.

Male sexual behavior was maintained at control levels by 215 and 430 mm^2 testosterone implants (Table VIII), in these same intact rabbits. Accessory sex organ weights and secretory activity were not in excess of the accessory sex gland weights and secretory activity of intact control rabbits. The fact that there was no significant difference in peripheral plasma testosterone concentration of rabbits receiving PDS cholesterol implants and those receiving 215 or 430 mm^2 testosterone implants supports these results.

The fact that testosterone was maintained at the same level in intact male rabbits from two sources (PDS testosterone implants and testes) suggests that the testicular contribution was decreased in animals receiving the 215 and 430 mm^2 PDS testosterone implants, respectively. This hypothesis is supported by the fact that sperm production and testis size in animals receiving 430 mm^2 PDS testosterone implants was significantly smaller than those receiving 215 mm^2 testosterone implants. An explanation for these results is that the hypophyseal production of interstitial cell stimulating hormone was reduced in animals receiving 215 mm^2 implants and further reduced in animals receiving 430 mm^2 implants, resulting in decreased testosterone secretion by Leydig cells and reduced spermatogenesis in both groups.

The fact that only 5 of 7 rabbits receiving the 430 mm^2 testosterone capsules were azoospermic suggests that larger implants are required to completely inhibit spermatogenesis in all rabbits. Therefore, experiment 5 was designed to answer the question of what size PDS testosterone implant would result in zero sperm production in 100% of the experimental rabbits without increasing accessory sex organ weights.

Rabbits receiving 800 mm^2 (wall thickness 0.245 mm) testosterone filled capsules had zero sperm production at 90 days post-implantation (Table XII). Moreover, seminal vesicle, prostate, vesicular and bulbourethral gland weights were not altered significantly in rabbits receiving 800 mm^2 PDS testosterone capsules. However, the fact that rabbits receiving capsules with a total of 1600 mm^2 had increased accessory sex organ weights shows the animal model selected for this study is sensitive to the androgenic effect of excessive dosages of testosterone.

This method of inhibition of spermatogenesis meets certain of the criteria for a systemic contraceptive agent listed in Chapter II. In rabbits the testosterone filled PDS implants have been shown to be 100% effective in producing aspermatogenesis, they are not deleterious to the accessory sex organs or male sexual behavior of recipient rabbits and a simple method for chronic administration of a readily available, economically produced material has been demonstrated. The specific criteria not investigated during this study are whether the antispermatogenic effect in rabbits is reversible thus what, if any, deleterious effects may be produced in offspring of recipients of PDS testosterone implants. Although extensive investigation remains to be performed in other species before intelligent predictions can be made for the use of PDS testosterone implants in humans as a male contraceptive, the present study is a significant contribution to the quest for an efficient, safe, systemic male contraceptive.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Appropriate androgen therapy (Heller et al., 1950) results in azoospermia in man that is reversible upon withdrawal of the hormonal steroid. It has been impractical to investigate the potential of this concept as a male contraceptive technique because of the difficulty in sustaining specific concentrations of androgens in peripheral blood over prolonged time periods. A particularly promising approach to administering androgens at controlled rates may involve subcutaneous implantation of androgen filled polydimethylsiloxane capsules. Numerous investigations have shown that androgens and other steroidal hormones pass through these polymers at predictable rates (Dziuk and Cook, 1966; Kincl, et al., 1968; Moon and Bunge, 1968; and Hwang et al., 1970).

Experiments have been completed demonstrating that testosterone tritium passses out of polydimethylsiloxane capsules suspended in water, that the tritiated material released into water has the same chromatographic mobility as authentic testosterone when subjected to thin-layer chromatography, that release rate into water (ug/24 hours) is dependent upon capsule surface area, that release from subcutaneous implants depends upon surface area and that release in vivo is constant over 3 months.

Castration abolished libido and caused accessory sex organ atrophy

in male rabbits. In contrast, libido and accessory sex organ weight and secretion in castrate rabbits receiving subcutaneous testosterone filled implants (430 mm^2) were identical to intact rabbits receiving subcutaneous cholesterol implants for 3 months. Intact rabbits containing subcutaneous testosterone filled implants (430 mm^2) for 3 months were similar to control rabbits in sex behavior score, concentrationn of testosterone in peripheral blood, accessory sex organ weight and seminal fructose and citric acid concentration. In contrast, paired testis weights and daily sperm production were significantly reduced in rabbits receiving the 430 mm^2 subcutaneous implants. However, only five of seven rabbits in this latter group were azoospermic. Subsequent experiments showed that 800 mm^2 subcutaneous testosterone filled polydimethylsiloxane implants resulted in azoospermia in all animals without causing accessory sex organ hypertrophy.

In conclusion the results of experiments 1-3 proved that testosterone-tritium passes through polydimethylsiloxane capsules suspended in water, that the tritiated material released into water has the same chromatographic mobility as authentic testosterone when subjected to thin-layer chromatography, that release rate into water is dependent upon capsules surface area, that release from capsules implanted either subcutaneously or intraperitoneally is similar and dependent upon capsule surface area.

The results of experiment four and five proved that subcutaneous polydimethylsiloxane testosterone implants totaling 800 mm^2 of surface area releases adequate amounts of testosterone to result in azoospermia in all of the experimental animals without causing hypertrophy of accessory sex organs.

These results suggest that the appropriate sized testosterone polydimethylsiloxane implant in the male rabbit will cause sterility for at least 3 months without causing accessory sex organ hypertrophy. Whether this concept will prove effective in the human remains to be determined.

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APPENDIX A

TABLE XIV

DISTRIBUTION OF TRITIUM IN THE PERIPHERAL PLASMA OF INTACT MALE RABBITS CONTAINING SUBCUTANEOUS PDS TESTOSTERONE-TRITIUM FILLED IMPLANTS[1]

Capsule Surface Area (mm^2)	Total Tritium in Plasma Sample	Total Tritium in Dichloromethane Extract		Testosterone Tritium	
	dpm/sample	dpm/sample	% of total tritium	dpm/sample	% of total tritium
225	5733	108	2	109	1.9
450	13514	275	2	160	1.2
900	14384	750	5.2	187	1.3

[1]The plasma from the 5th week of blood collection reported in Table II was pooled. A 1 ml aliquot was removed from pooled plasma and counted in Aquasol (New England Nuclear). The remainder of the plasma pool was extracted three times with 2.5 volumes of dichloromethane. The aliquot was removed and counted in Aquasol as above. The remainder was concentrated under nitrogen, developed in thin layer chromatography system benzene:ethyl acetate (110:100) and the area corresponding chromatographically to testosterone eluted and counted in Aquasol. All counts were corrected for quenching, loss through the respective steps and for aliquot losses.

APPENDIX B

TABLE XV

ANALYSIS OF VARIANCE[1] OF TESTOSTERONE CONCENTRATION IN DISTILLED WATER BATHING PDS[2] TESTOSTERONE CAPSULES

Source	df	ss	ms	F Ratio[3]
Total (uncor)	36		--	--
Mean	1	368,362	--	--
Surface Area (SA)	2	122,455	61,228	27.9*
Time (t)	2	51,280	25,640	62.8*
SA x T	4	8,787	2,197	5.4*
Error	27	11,017	408	

1. A two factor analysis, completely randomized design, consider SA fixed and T random.

2. Polydimethylsiloxane.

3. F Ratios: for SA use SA/SA x T; for T use T/EMS; for SA x T use SA x T/EMS.

*P<0.005.

TABLE XVI

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO TESTOSTERONE CONCENTRATIONS IN DISTILLED WATER BATHING PDS TESTOSTERONE CAPSULES

		p=2	p=3	p=4	p=5	p=6	p=7	p=8	p=9
		Critical Values, p<0.05							
Trt. Code	Ranked Means	23.9	25.0	25.7	26.4	26.8	27.1	27.4	27.9
900-24	212								
900-12	138	74							
900-6	96	42	116						
450-24	96	0	42	116					
225-24	62	34	34	76	150				
450-12	53	9	43	43	85	159			
225-12	33	20	29	63	63	105	179		
450-6	32	1	21	30	64	64	106	180	
225-6	20	12	13	33	42	76	76	118	192

TABLE XVII

ANALYSIS OF VARIANCE[1] OF TRITIUM CONCENTRATION IN PERIPHERAL PLASMA OF INTACT MALE RABBITS CONTAINING SUBCUTANEOUS PDS[2] TESTOSTERONE CAPSULES

	Experiment 2			
Source	df	ss	ms	F Ratio[3]
Total (uncor.)	126	--	--	--
Mean	1	17,327,929	--	--
Surface Area (SA)	2	3,768,222	1,884,111	159.8*
Time (T)	5	83,087	16,617	<1
SA x T	10	117,903	11,790	<1
Error	108	9,878,874	91,471	

1. A two factor analysis, completely randomized design, consider SA fixed and T random.
2. Polydimethylsiloxane
3. F ratios: for SA use SA/SA x T; for T use T/EMS; for SA x T use SA x T/EMS.

*$P<0.005$.

TABLE XVIII

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO TRITIUM CONCENTRATION IN PERIPHERAL PLASMA OF INTACT MALE RABBITS CONTAINING SUBCUTANEOUS PDS TESTOSTERONE CAPSULES: EXPERIMENT 4

Trt. Code	Ranked Means	p=2	p=3	p=4	p=5	p=6	p=7	p=8	p=9	p=10	p=11	p=12	p=13	p=14	p=15	p=16	p=17	p=18	p=19	p=20	p=21
		Critical Values, p<0.05																			
		76	80	82	84	86	87	88	89	89	90	90	91	91	92	92	92	93	93	93	94
900-1	797																				
900-4	746	51																			
900-2	741	5	56																		
900-5	723	17	23	74																	
900-3	690	33	51	56	107																
900-6	687	3	36	54	59	110															
900-7	661	26	29	62	80	85	136														
450-2	350	11	337	340	373	391	396	447													
450-6	347	3	314	340	343	376	394	399	450												
450-1	331	16	19	330	356	359	392	410	415	466											
450-3	307	24	40	43	354	380	383	416	434	439	490										
450-7	277	30	54	70	73	384	410	413	446	464	469	520									
225-1	269	8	38	62	78	81	392	418	421	454	472	477	528								
225-2	256	13	21	51	75	91	94	405	431	434	467	485	490	541							
450-5	252	4	17	25	55	79	95	98	409	437	438	471	489	494	545						
450-4	250	2	6	19	27	57	81	97	100	411	437	440	473	491	496	547					
225-3	196	54	56	60	73	81	111	135	151	154	465	491	494	427	545	550	601				
225-7	182	14	68	70	70	87	95	125	149	165	168	479	505	508	541	559	564	615			
225-6	181	1	15	69	69	88	88	96	126	150	156	179	480	506	509	542	560	565	616		
225-5	162	19	20	34	34	94	94	107	115	145	169	185	188	499	522	528	561	579	584	635	
225-4	152	10	29	30	30	98	100	104	117	125	155	179	195	198	509	535	538	571	589	594	645

TABLE XIX

ANALYSIS OF VARIANCE[1] OF PERIPHERAL PLASMA TRITIUM CONCENTRATION OF INTACT MALE RABBITS CONTAINING SUBCUTANEOUS OR INTRAPERITONEAL 450 SQ. MM. PDS[2] TESTOSTERONE IMPLANTS: EXPERIMENT 3

Source	df	ss	ms	F Ratio
Total (cor.)	59	556,476	--	--
Between	1	5,153	5,153	<1
Within	58	551,323	9,506	

1. One-way analysis of variance.
2. Polydimethylsiloxane.

TABLE XX

ANALYSIS OF VARIANCE[1] OF PERIPHERAL PLASMA TRITIUM CONCENTRATION OF INTACT MALE RABBITS CONTAINING SUBCUTANEOUS OR INTRAPERITONEAL 900 SQ. MM. PDS[2] TESTOSTERONE IMPLANTS: EXPERIMENT 3

Source	df	ss	ms	F Ratio
Total (cor.)	59	2,198,972	--	--
Between	1	122,221	122,221	3.41
Within	58	2,076,751	35,806	

1. One-way analysis of variance.
2. Polydimethylsiloxane.

TABLE XXI

ANALYSIS OF VARIANCE[1] OF TOTAL DAILY SPERM PRODUCTION OF RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio
Total (cor.)	20	208,729	--	--
Between	2	138,668	69,334	17.81*
Within	18	70,062	3,892	

1. One-way analysis of variance.
*P<0.01.

TABLE XXII

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO TOTAL DAILY SPERM PRODUCTION OF RABBITS: EXPERIMENT 4

Treatment Code	Ranked Means	p=2 Critical Values, p<0.05 69.56	p=3 Critical Values, p<0.05 73.1
430 C Intact	214.9		
215 T Intact	123.5	91.4	
430 T Intact	16.1	107.4	198.3

TABLE XXIII

ANALYSIS OF VARIANCE[1] OF PAIRED TESTIS WEIGHTS OF RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio
Total (cor.)	20	100.5156		
Between	2	73.5606	36.7803	24.52*
Within	18	26.9550	1.50	

1. One-way analysis of variance.
*P<0.01.

TABLE XXIV

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO PAIRED TESTIS WEIGHTS OF RABBITS: EXPERIMENT 4

Treatment Code	Ranked Means	p=2 Critical Values, P<0.05 1.37	p=3 Critical Values, P<0.05 1.44
430 C Intact	6.14		
215 T Intact	3.62	2.52	
430 T Intact	1.56	2.06	4.58

TABLE XXV

ANALYSIS OF VARIANCE[1] OF SEX BEHAVIOR SCORES OF RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (cor.)	41	732.61		
Implant (I)	2	265.43	132.7	2.67
Testes (T)	1	107.52	107.5	14.87*
I x T	2	99.56	49.8	6.89
Error	36	260.1	7.3	

1. A two factor analysis, completely randomized design, consider I fixed and T random.

2. F ratios: for I use I/I x T; for T use T/EMS; for I x T use I x T/EMS.

*P<0.01.

TABLE XXVI

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO SEX BEHAVIOR SCORES OF RABBITS: EXPERIMENT 4

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		2.95	3.1	3.18	3.26	3.32
430 T Intact	9.9					
430 T Cast.	9.8	.1				
430 C Intact	8.65	1.15	1.25			
215 T Intact	8.07	.58	1.73	1.83		
215 T Cast.	6.03	2.04	2.62	3.77	3.87	
430 C Cast.	0.0	6.03	8.07	8.65	9.8	9.9

TABLE XXVII

ANALYSIS OF VARIANCE[1] OF TESTOSTERONE CONCENTRATION IN PERIPHERAL PLASMA OF MALE RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (Cor.)	35	7416.97		
Treatment	5	2126.30	425.3	
Implant (I)	2	509.68	254.8	<1
Testes (T)	1	86.18	86.2	<1
I x T	2	1530.41	765.2	4.34
Error	30	5290.70	176.4	

1. A two factor analysis, completely randomized design, consider I fixed and T random.

2. F ratios: for I use I/I x T; for T use T/EMS; for I x T use I x T/EMS.

*P<0.05.

TABLE XXVIII

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO TESTOSTERONE CONCENTRATION IN PERIPHERAL PLASMA OF MALE RABBITS: EXPERIMENT 4

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		15.66	16.48	16.9	17.3	17.6
430 T Cast.	2.24					
430 C Intact	2.14	1.0				
215 T Cast.	2.09	1.5	.50			
430 T Intact	1.66	5.8	4.80	4.3		
215 T Intact	1.45	7.9	6.9	6.4	2.1	
430 C Cast.	0.0	22.4	21.4	20.9	16.6	14.5

TABLE XXIX

ANALYSIS OF VARIANCE[1] OF SEMINAL VESICLE WEIGHTS OF RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (cor.)	41	1.364	--	--
Implant (I)	2	.122	.061	<1
Testes (T)	1	.132	.132	6.67*
I x T	2	.398	.199	10.05**
Error	36	.712	.019	

1. A two factor analysis, completely randomized design, consider I fixed and T random.

2. F ratios: for I use I/I x T; for T use T/EMS; for I x T use I x T/EMS.

*P<0.025.

**P<0.005.

TABLE XXX

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO SEMINAL VESICLE WEIGHTS OF RABBITS: EXPERIMENT 4

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		150	158	162	166	169
430 C Intact	508					
430 T Cast.	476	32				
430 T Intact	398	78	110			
215 T Cast.	358	40	118	150		
215 T Cast.	297	61	101	179	211	
430 C Cast.	135	162	223	263	341	373

TABLE XXXI

ANALYSIS OF VARIANCE[1] OF PROSTATE GLAND WEIGHTS OF RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (cor.)	41	1,831.9	--	--
Implant (I)	2	145.3	72.7	<1
Testes (T)	1	367.9	367.9	27.05*
I x T	2	829.7	414.9	30.5*
Error	36	488.0	13.6	

1. A two factor analysis, completely randomized design, consider I fixed and T random.

2. F ratios: for I use I/I x T; for T use T/EMS; for I x T use I x T/EMS.

*P<0.005.

TABLE XXXII

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO PROSTATE GLAND WEIGHTS OF RABBITS: EXPERIMENT 4

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		125.7	132.2	135.7	139.2	141.4
430 C Intact	693					
430 T Cast.	572	121				
430 T Intact	510	183	62			
215 T Intact	458	235	114	52		
215 T Cast.	415	278	157	92	43	
430 C Cast.	113	580	457	397	345	0.302

TABLE XXXIII

ANALYSIS OF VARIANCE[1] OF VESICULAR GLAND WEIGHTS OF RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (cor.)	41	3,902.6	--	--
Implant (I)	2	735.2	367.6	<1
Testes (T)	1	991.1	997.1	25.81*
I x T	2	795.4	397.7	10.36*
Error	36	1,308.9	38.4	

1. A two factor analysis; completely randomized design, consider I fixed and T random.

2. F ratios: for I use I/ I x T; for T use T/EMS; for I x T/EMS.

*P<0.005.

TABLE XXXIV

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO VESICULAR GLAND WEIGHTS OF RABBITS: EXPERIMENT 4

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		214	225	231	237	241
430 C Intact	807					
430 T Intact	804	3				
430 T Cast.	778	29	26			
215 T Intact	762	45	42	16		
215 T Cast.	539	268	265	239	223	
430 C Cast.	129	678	675	649	633	410

TABLE XXXV

ANALYSIS OF VARIANCE[1] OF BULBOURETHRAL GLAND WEIGHTS OF RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (cor.)	41	1,755.8	--	--
Implants (I)	2	275.5	137.8	<1
Testes (T)	1	295.7	295.7	12.32*
I x T	2	322.1	161.1	6.71*
Error	36	862.5	24	

1. A two factor analysis, completely randomized design, consider I fixed and T random.

2. F ratio: for I use I/I x T; for T use T/EMS; for I x T use I x T/EMS.

*P<0.005.

TABLE XXXVI

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO BULBOURETHRAL GLAND WEIGHTS OF RABBITS: EXPERIMENT 4

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		619	177	182	187	190
430 C Intact	622					
430 T Cast.	616	6				
430 T Intact	608	14	8			
215 T Intact	527	95	89	81		
215 T Cast.	424	198	192	184	103	
430 C Cast.	216	406	40	392	311	208

TABLE XXXVII

ANALYSIS OF VARIANCE[1] OF CITRIC ACID CONCENTRATION IN RABBIT SEMINAL PLASMA: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (cor.)	41	32.7464	--	--
Implant (I)	2	7.5412	7.54	1.68
Testes (T)	1	5.9853	5.99	21.1*
I x T	2	9.0065	4.50	15.9*
Error	36	10.2134	0.2837	

1. A two factor analysis, completely randomized design, consider I fixed and T random.

2. F ratios: for I use I/I x T; for T use T/EMS; for I x T use I x T/EMS.

*P<0.005.

TABLE XXXVIII

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO CITRIC ACID CONCENTRATION IN RABBIT SEMINAL PLASMA: EXPERIMENT 4

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		0.5815	0.611	0.628	0.644	0.654
430 T Cast.	2.11					
430 C Intact	1.73	0.38				
215 T Intact	1.64	0.09	0.47			
430 T Intact	1.62	0.02	0.11	0.49		
215 T Cast.	0.62	1.00	1.02	1.11	1.49	
430 C Cast	0.0	0.62	1.62	1.64	1.73	2.11

TABLE XXXIX

ANALYSIS OF VARIANCE[1] OF FRUCTOSE CONCENTRATION IN RABBIT SEMINAL PLASMA: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (cor.)	41	--	--	--
Implants (I)	2	6.3317	3.1659	<1
Testes (T)	1	10.6304	10.6304	15.47*
I x T	2	16.8905	8.4453	12.29*
Error	36	24.744	0.6873	

1. A two factor analysis; completely randomized design, consider I fixed and T random.

2. F ratios: for I use I/IxT; for T use T/EMS; for I x T use I xT/EMS.

*P<0.005.

TABLE XL

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO FRUCTOSE CONCENTRATION IN RABBIT SEMINAL PLASMA: EXPERIMENT 4

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		0.905	0.952	0.978	1.00	1.02
430 T Cast.	2.46					
215 T Intact	2.37	0.90				
430 C Intact	2.30	0.70	0.160			
430 T Intact	1.74	0.56	0.630	0.72		
215 T Cast.	0.928	0.812	1.37	1.44	1.53	
430 C Cast.	0.0	0.928	1.74	2.30	2.37	2.46

TABLE XLI

ANALYSIS OF VARIANCE[1] OF TOTAL DAILY SPERM PRODUCTION IN MATURE RABBITS: EXPERIMENT 4

Source	df	ss	ms	F Ratio[2]
Total (cor.)	35	448,730	--	--
Treatment	5	284,893	56,978.6	10.43*
Error	30	163,837	5,461	

1. One-way analysis of variance.
*P<0.005.

TABLE XLII

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO TOTAL DAILY SPERM PRODUCTION IN MATURE RABBITS: EXPERIMENT 5

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		35.5	37.3	38.3	39.3	39.9
400 C	210					
100 T	198	12				
200 T	154	44	56			
400 T	34	120	164	176		
1600 T	7	27	147	191	203	
800 T	0.0	7	34	154	198	210

TABLE XLIII

ANALYSIS OF VARIANCE[1] OF PAIRED TESTIS WEIGHTS OF MATURE RABBITS: EXPERIMENT 5

Source	df	ss	ms	F Ratio
Total (cor.)	35	159.83	--	--
Treatment	5	120.93	24.2	18.60*
Error	30	38.9	1.3	

1. One-way analysis of variance.
*P<0.005.

TABLE XLIV

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO PAIRED TESTIS WEIGHTS OF MATURE RABBITS: EXPERIMENT 5

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		0.55	0.53	0.59	0.61	0.62
400 C	4.9					
100 T	4.9	0.0				
200 T	4.5	0.4	0.4			
400 T	1.9	2.6	3.0	3.0		
1600 T	1.0	0.9	3.5	3.9	3.9	
800 T	.7	0.3	1.2	2.8	4.2	4.2

TABLE XLV

ANALYSIS OF VARIANCE[1] OF SEMINAL VESICLE WEIGHTS OF MATURE RABBITS: EXPERIMENT 5

Source	df	ss	ms	F Ratio
Total (cor.)	35	3,416	--	--
Treatment	5	2,419	483.9	14.66*
Error	30	987	33	

1. One-way analysis of variance.
*P<0.005.

TABLE XLVI

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO SEMINAL VESICLE WEIGHTS OF MATURE RABBITS: EXPERIMENT 5

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		87	90	94	96	98
1600 T	1,015					
200 T	389	626.5				
800 T	375	14.0	62.05			
400 C	312	62.4	76.4	702.9		
400 T	311	0.8	63.2	77.2	693.7	
100 T	306	5.0	5.8	68.2	82.2	709

TABLE XLVII

ANALYSIS OF VARIANCE[1] OF VESICULAR GLAND WEIGHTS OF MATURE RABBITS: EXPERIMENT 5

Source	df	ss	ms	F Ratio
Total (cor.)	35	1,755,095	--	--
Treatment	5	569,264	113,853	2.88*
Error	30	1,185,831	39,528	

1. One-way analysis of variance.
*P<0.05.

TABLE XLVIII

DUNCAN'S NEW MULTIPLE RANGE TEST APPLIED TO VESICULAR GLAND WEIGHTS OF MATURE RABBITS: EXPERIMENT 5

Treatment Code	Ranked Means	p=2	p=3	p=4	p=5	p=6
		Critical Values, P<0.05				
		235	247	253	260	264
1600 T	955					
100 T	788	167				
800 T	710	79	245			
200 T	652.0	58	136	303		
400 C	642	10	68	147	313	
400 T	565	77	97	145	224	390

TABLE XLIX

ANALYSIS OF VARIANCE[1] OF PROSTATE GLAND WEIGHTS OF INTACT MALE RABBITS: EXPERIMENT 5

Source	df	ss	ms	F Ratio
Total (cor.)	35	1,520,000	--	--
Treatment	5	227,000	45,400	1.05
Error	30	1,293,000	43,100	

1. One-way analysis of variance.

TABLE L

ANALYSIS OF VARIANCE[1] OF BULBOURETHRAL GLAND WEIGHTS OF INTACT RABBITS: EXPERIMENT 5

Source	df	ss	ms	F Ratio
Total (cor.)	35	2,531,662		
Treatment	5	566,840	113,368	1.73
Error	30	1,964,822	65,494	

1. One-way analysis of variance.

VITA

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